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Intra-articular hyaluronan treatment and its mechanism of action in relieving inflammation and pain in equine arthritis

*Clinical and experimental trials on treatment efficacy of hyaluronan
and its effects on synovial fluid biomarkers*

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ACADEMIC DISSERTATION

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To my Family

Abstract

Intra-articular (IA) inflammation resulting in lameness is a common health problem in horses. Exogenous IA hyaluronic acid has been shown to provide an analgesic effect and reduce pain in equine and human osteoarthritis. High molecular weight non-animal stabilized hyaluronic acid (NASHA) has gained popularity in the treatment of human arthritic conditions due to its long-acting pain-relieving effects. In animal models, hyaluronan has been demonstrated to reduce pain by protecting nociceptive nerve endings and blocking pain receptor channels.

Inflammatory and degenerative activity inside the joint can be studied *in vivo* by analysis of synovial fluid (SF) biomarkers. In addition to pro-inflammatory mediators, several anabolic and anti-inflammatory substances are produced during the disease process. They counteract the catabolic effects of pro-inflammatory cytokines, thus diminishing cartilage damage. The response of SF biomarkers after IA hyaluronan injection, alone or in combination with other substances, has been examined only in a few equine studies.

Our aim was to examine the efficacy of IA NASHA in relieving lameness and related signs of IA inflammation in clinical and experimental studies. We also investigated the effect of NASHA on various SF biomarkers. In addition, we explored possible adverse effects of NASHA on clinical signs of IA inflammation and SF.

In the clinical study, we compared the response to treatment of lameness localized in the equine metacarpophalangeal joint injected with NASHA or placebo (saline). We also investigated the response of SF prostaglandin E₂ (PGE₂), substance P, aggrecan chondroitin sulphate 846 epitope (CS846), and carboxypeptide of type II collagen (CPII) concentrations to the NASHA and placebo treatments. Thirty clinically lame horses with a positive response to diagnostic IA anaesthesia of the metacarpophalangeal joint and with no, or at most mild radiographic changes in this joint were included in the study. After collection of baseline SF samples followed by IA diagnostic anaesthesia, horses in the treatment group received 3 ml of a NASHA product (20 mg/ml) intra-articularly, and those in the placebo group received an equivalent volume of sterile 0.9% saline solution. The horses were re-evaluated and a second SF sample was obtained after a two-week period.

In the experimental study, the aim was to measure the SF interleukin-1 receptor antagonist (IL-1ra), platelet-derived growth factor BB (PDGF-BB), transforming growth factor beta 1 (TGF- β_1), and tumour necrosis factor alpha (TNF- α) concentrations before and after surgically induced cartilage defect and sham operation as a control in horses. We also investigated whether the concentrations of selected biomarkers in SF changed following NASHA injection.

Our results indicate that a single IA NASHA injection is not better than a single saline injection for reducing lameness in horses with synovitis or mild osteoarthritis. However, IA NASHA may have some beneficial effects in modifying mild clinical signs. The

decrease in the SF concentration of the cartilage-derived biomarker CS846 only in the NASHA group suggests that less damage and hence less repair to the cartilage has occurred post-injection. Creation of the cartilage defect and sham operation lead to an increase of synovial fluid IL-1ra and TNF- α concentrations in the experimental study, but NASHA failed to produce changes in SF biomarkers. Further research is needed to document function of anabolic growth factors in equine arthritis as well as possible treatment effects of IA hyaluronan on the SF biomarkers of inflammation and cartilage metabolism.

The significant increase in SF white blood cell count after IA NASHA may indicate a mild inflammatory response. However, as no clinical adverse effects were observed, IA NASHA appeared to be well tolerated.

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Contents

Abstract	iv
Acknowledgements	vi
List of original publications	11
Abbreviations	12
1 Introduction	13
2 Review of the literature	15
2.1 Arthritis and osteoarthritis	15
2.2 Intra-articular treatment of joint pain in horses	17
2.2.1 Hyaluronan	17
2.2.2 Corticosteroids, polysulphated glycosaminoglycans, and autologous conditioned serum	20
2.3 Molecular mediators as biomarkers in intra-articular inflammation and cartilage metabolism	21
2.3.1 Inflammatory mediators	22
2.3.2 Markers of cartilage metabolism	24
2.3.3 Interleukin-1 receptor antagonist	25
2.3.4 Anabolic growth factors	25
3 Aims of the thesis	27
4 Materials and methods	28
4.1 Approval of study protocols	28
4.2 Study populations	28
4.2.1 Clinical patients (I,II)	28
4.2.2 Experimental horses (III)	30
4.3 Study design	31
4.3.1 Clinical study (I,II)	31

4.3.2 Experimental study (III)	32
4.4 Examination procedures	33
4.4.1 Clinical study: baseline clinical examination (I,II)	33
4.4.2 Clinical study: second clinical examination (I,II)	36
4.4.3 Experimental study: lameness examinations, surgical procedures, synovial fluid sampling, and IA injection (III)	36
4.5 Laboratory analyses	38
4.6 Outcome measures	41
4.6.1 Clinical study (I,II)	41
4.6.2 Experimental study (III)	41
4.7 Statistical analyses	41
4.7.1 Clinical study (I,II)	41
4.7.2 Experimental study (III)	43
5 Results	44
5.2 Hyaluronan injection for treatment of pain originating from the metacarpophalangeal joint (I)	44
5.2.1 Demographic variables and outcome measures at the baseline clinical examination	44
5.2.2 Outcome measures after NASHA and placebo injection	44
5.2.3 Follow-up	45
5.3 Experimental study: Clinical outcomes of experimental horses (III)	46
5.4 Synovial fluid biomarkers in arthritis and following NASHA injection (II,III)	48
6 Discussion	54
6.1 NASHA injection for treatment of intra-articular pain	54
6.2 Tolerability of NASHA	55
6.3 Biomarker analyses	56
6.3.1 Effect of NASHA on synovial fluid biomarkers in lameness originating from the metacarpophalangeal joint (II)	56

6.3.2 Effect of induced cartilage defect and NASHA on synovial fluid biomarkers (III)	58
6.4 Study limitations	60
6.4 Future perspectives	64
7 Conclusions	66
References	67

List of original publications

This thesis is based on the following publications:

- I Niemelä TM, Tulamo R-M, Hielm-Björkman AK, 2016. A randomised, double-blinded, placebo-controlled clinical study on intra-articular hyaluronan treatment in equine lameness originating from the metacarpophalangeal joint. *BMC Veterinary Research*. 12, 60.
- II Niemelä TM, Tulamo R-M, Aaltonen K, Sankari SM, Hielm-Björkman AK, 2018. Changes in biomarkers in equine synovial fluid two weeks after intra-articular hyaluronan treatment: a randomised double-blind clinical trial. *BMC Veterinary Research*. 14, 186.
- III Niemelä TM, Tulamo R-M, Carmona JU, López C, 2019. Evaluation of the effect of experimentally induced cartilage defect and intra-articular hyaluronan on synovial fluid biomarkers in intercarpal joints of horses. *Acta Veterinaria Scandinavica*. 61, 24.

The publications are referred to in the text by their Roman numerals and have been reprinted with the permission of their copyright holders.

Abbreviations

ACS	Autologous conditioned serum
CD	Cartilage defect
CPII	Carboxypeptide of type II collagen
CS	Chondroitin sulphate
CS846	Aggrecan chondroitin sulphate 846 epitope
CV	Coefficient of variation
DLPM	Dorsolateral-palmaromedial
DMOAD	Disease-modifying osteoarthritic drug
DMPL	Dorsomedial-palmarolateral
DP	Dorsopalmar
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
GAG	Glycosaminoglycan
HA	Hyaluronic acid, also known as hyaluronan
IA	Intra-articular
IL-1ra	Interleukin-1 receptor antagonist
IQR	Interquartile range
LM	Lateromedial
LPS	Lipopolysaccharide
MCPJ	Metacarpophalangeal joint
MTPJ	Metatarsophalangeal joint
MW	Molecular weight
NASHA	Non-animal stabilized hyaluronic acid
NSAID	Non-steroidal anti-inflammatory drug
OA	Osteoarthritis
PDGF-BB	Platelet-derived growth factor BB
PGE ₂	Prostaglandin E ₂
PRP	Platelet-rich plasma
PSGAG	Polysulphated glycosaminoglycan
RT-PCR	Reverse transcription polymerase chain reaction
SF	Synovial fluid
SO	Sham-operation
TGF- β_1	Transforming growth factor beta 1
TNF- α	Tumour necrosis factor alpha
TP	Total protein
WBC	White blood cell

1 Introduction

Over 70% of racehorses suffer from lameness during their lifetime and the cause is often intra-articular (IA) inflammation (Morris and Seeherman, 1991). Lameness resulting from a joint disease is one of the major causes of poor performance in horses (Jeffcott et al., 1982; Morris and Seeherman, 1991). IA inflammation may lead to the development of chronic osteoarthritis (OA). This condition can remain silent for a long period, only becoming clinically and radiographically evident in advanced OA (McIlwraith, 1997). Since OA often ends the sports career of the horse, early detection and treatment as well as prevention of further articular damage are important.

Hyaluronic acid (HA), also known as hyaluronan, is a widely used IA treatment in equine joint diseases and has been in use since the early 1970s. However, the efficacy and mechanism of action of HA have seldom been studied in horses, especially outside experimental settings. High-molecular weight (MW) HA may be superior in the treatment of IA inflammation and pain. Non-animal stabilized hyaluronic acid (NASHA) is a cross-linked HA with a large MW (Adams, 1993). NASHA has a long half-life, thus remaining in the synovial structures for a considerably longer period than other products (Lindqvist et al., 2002). A duration of treatment effect of up to six months has been reported in human patients (Åkermark et al., 2002; Altman et al. 2004). The long residence time is attributable to the stabilized network of HA molecules within NASHA. The long-lasting effect is a desirable feature especially in the IA treatment of horses. Recurrent IA injections predispose to intra-synovial infections as well as to adverse effects of HA, such as severe effusion and pain in the joint, which have been encountered relatively often in human joints (Altman et al., 2004; Marino et al., 2006). In horses, recurrent lameness and withdrawal periods for injections due to antidoping regulations cause longer breaks in the horses' sports careers. Currently, no research on NASHA in treatment of equine IA inflammation has been published besides the present study.

Biomarkers are molecular markers of inflammatory processes and tissue turnover. Ideally, inflammatory and degenerative activity inside the joint can be monitored via analysis of synovial fluid (SF) biomarkers. Research of the biomarkers in equine SF has aimed at finding tools for early diagnosis of OA and for monitoring progression of the condition. Some SF biomarkers, such as prostaglandin E₂, have been documented especially in experimental equine studies (Owens et al., 1996; Kawcak et al., 1997; Schleining et al., 2008; Frisbie et al., 2009a; Frisbie et al., 2009b; de Grauw et al., 2009a; de Grauw et al., 2009b; van Loon et al., 2010; Frisbie et al., 2013). Others, particularly anti-inflammatory cytokines, warrant further research. The purely catabolic cytokine TNF- α has been evaluated in several equine studies (Billinghurst et al., 1995; Hawkins et al., 1995; van den Boom et al., 2004; Kamm et al., 2010; Carmalt et al., 2011; Ríos et al., 2015a; Textor et al., 2013; Ma et al., 2017), but IL-1ra, PDGF-BB, and TGF- β 1 measured in the present study have been evaluated each only in a few studies on equine SF (Frisbie et al., 2007; Carmalt et al., 2011; de Grauw et al., 2011; Textor et al., 2013) and

simultaneously only by Ríos et al. (2015a). More research using clinical patients with naturally occurring joint disease is needed to define more closely the change of the biomarker concentrations in SF, especially in relation to the early stage of the disease.

The aim of this study was to investigate the efficacy of IA NASHA on the clinical signs of arthritis and early osteoarthritis. A second objective was to explore selected pro-inflammatory, anti-inflammatory, and cartilage metabolism markers in the SF of naturally occurring mild arthritis and in acute IA inflammation caused by surgically induced cartilage defect in the horse. Furthermore, the short-term response of the selected SF biomarkers after IA NASHA treatment was investigated.

2 Review of the literature

2.1 Arthritis and osteoarthritis

Aetiology and pathophysiology

Damage to the articular cartilage, subchondral bone, synovium, joint capsule, or other soft tissues, such as collateral ligaments, and subsequent inflammatory arthritis may lead to degeneration of the joint (Buckwalter and Brown, 2004). Arthritis resulting from acute or repetitive overload injuries of the joint, also known as synovitis, can result in the synthesis of defective cartilage matrix components, leading eventually to OA (McIlwraith, 2011a). In effect, primary traumatic synovitis can be considered an early form of OA (Bertone, 2011), which is defined as a disorder of movable joints characterized by degeneration and loss of articular cartilage (Frisbie et al., 2012). In addition to the degeneration of cartilage, also abnormal bone remodelling, osteophyte formation, and joint inflammation accompany OA (Kraus et al., 2015), and they may all contribute to the generation and maintenance of pain (Perrot, 2015).

Despite comprehensive research, the aetiology of OA is not fully understood. Repeated microtrauma, especially due to recurrent overload in use, is likely the most common cause of equine OA (Caron, 2011; Pinchbeck et al., 2013). Recent studies in human medicine point to an inflammatory mechanism in the initial stages of the disease. Whether the inflammation in the synovium is the primary phenomenon initiating the cartilage degeneration or whether the inflammation is a consequence of the changes in the cartilage is unresolved (Sutton et al., 2009; Sokolove and Lepus, 2013). Inflammation has been suggested to occur mainly in response to injury caused by mechanical stimulation of the joint (Man and Mologhianu, 2014; Glyn-Jones et al., 2015; Kraus et al., 2015). The increased number and nature of deregulated proteins observed in OA with respect to osteochondrosis have been considered to confirm the high inflammatory character of this disease in horses (Chiaradia et al., 2012).

However, the specific roles and interactions of various mechanical and biological factors contributing to the development of OA are unknown. According to one hypothesis, damage to the cartilage is a consequence of increased mechanical stress due to increased density of the subchondral bone plate and adjacent trabeculae (Radin and Rose, 1986; Norrdin et al., 1998). The increased density of the bone results from microfractures exceeding the healing and remodelling rate of the bone under normal mechanical stress. As a consequence, the bone has reduced ability to absorb repetitive physiological loads, which increases the stress directed to the cartilage (Radin and Rose, 1986). In horses, high levels of hard tissue microdamage have been found beneath the regions of intact hyaline cartilage in the condyles of the distal part of the third metacarpal bone. This supports the hypothesis that substantial disruption of the subchondral bone precedes cartilage

degeneration in the development of lesions. Degeneration in hyaline cartilage and calcified cartilage associated with focal microstructural changes deep in the underlying trabecular bone of the third carpal bone of horses suffering from post-traumatic osteoarthritis have also been reported (Lacourt et al., 2012; Bertuglia et al., 2016). Greater density of osteoclasts and bone porosity in the underlying bone were suggested to contribute to the degeneration of the calcified cartilage (Bertuglia et al., 2016).

The cartilage matrix can also be inherently defective and have abnormal biomechanical properties. This leads to failure of the cartilage under normal loading, resulting in OA (Katzenstein et al., 1990). However, OA resulting from inherently defective cartilage matrix components has not yet been identified in the horse (Caron, 2011). In general, clinical joint disease in horses usually involves multiple joint tissues with some degree of pathology (Frisbie et al., 2012). Instead of being a single disease, equine OA probably reflects a common response of joint tissues to several potential causes (Caron, 2011). Increasing age, female gender in the case of mild OA, training regimen, and increased number of races during a lifetime have been identified as risk factors for OA in horses (Reed et al., 2012; Pinchbeck et al., 2013; Turley et al., 2014).

The molecular level of OA and the mediators of IA inflammation and metabolism are introduced in Section 2.3.

Diagnosis of osteoarthritis in horses

Intra-articular inflammation and pain in horses are manifested as lameness, a gait abnormality. Lameness examination of horses aim at determination of the location of pain and lameness, prior to further evaluation with diagnostic imaging methods. Recently, objective methods for detecting lameness, such as three-dimensional optical motion capture (Rhodin et al., 2013) and inertial measurement units (Greve et al., 2017), have been shown to be useful in determining the lame limb. Localization of pain by regional as well as IA analgesia plays a prominent role in clinical lameness evaluation of horses (Bassage and Ross, 2011). IA analgesia is generally considered more specific for an IA localization of a painful process than perineural nerve blocks and is often used to discriminate between intra- and periarticular sources of pain (Nagy et al., 2009).

Radiography has long been used for assessing the structural changes of OA. However, it is of limited value in identifying horses with incipient or focal lesions (Caron, 2011), and a 30–40% change in bone mineral density is still needed to detect lesions in digital radiographs (Hunter et al., 2013). OA may remain asymptomatic for a long period and only become clinically and radiographically evident when the disease has progressed. Therefore, the correlation between the degree of radiological changes associated with OA and the degree of pain and lameness is relatively poor (Frisbie, 2012). A lack of correlation between arthroscopically evident degeneration and radiological findings has also been reported (Kannegieter and Burbridge, 1990; Moore and Schneider, 1995). Radiological findings associated with OA include periarticular osteophyte formation,

narrowing of the joint space, subchondral lucent zones, increased subchondral bone opacity (sclerosis), loss of trabecular pattern in subchondral bone, thickening of the subchondral bone plate, and joint capsule distention (Dyson, 2011).

2.2 Intra-articular treatment of joint pain in horses

Although the articular cartilage is devoid of innervation, the surrounding tissues are rich in unmyelinated C fibres. The joint capsule, synovium, tendons, ligaments, periosteum, and bone have dense innervation of unmyelinated C fibres and are sources of IA pain (Wyke, 1972). Sensory nerves respond both to mechanical stimuli, such as increased pressure due to SF effusion, and to chemical mediators. Some mediators, such as neuropeptides (e.g. substance P), stimulate pain fibres directly. These mediators, along with others, such as PGE₂ and IL-1, also sensitize fibres to be more reactive after mechanical stimulation (Frisbie, 2012).

The medical treatment of IA inflammation and pain is aimed at returning the joint to normal as quickly as possible and preventing the occurrence or reducing the severity of OA, thereby minimizing lameness and joint deterioration. Medications that alleviate pain, but do not have any therapeutic effect on the cartilage matrix are termed symptom-modifying osteoarthritic drugs (SMOADs). Disease-modifying osteoarthritic drugs (DMOADs) affect positively either the articular cartilage or the synovial environment (McIlwraith, 2011b).

2.2.1 Hyaluronan

General

Hyaluronan, also called hyaluronic acid (HA), is a GAG that has an important role in the formation of proteoglycan aggregates in cartilage. It is a component of the ECM and SF. Synovial membrane type B cells and chondrocytes synthesize HA by disaccharide oligomerization (Gadher and Woolley, 1987). In OA, the synthesis of HA is disrupted by increased levels of pro-inflammatory cytokines, free radicals, and proteinases, resulting in HA with a significantly reduced MW, more molecular polydisaccharides, and a reduction in SF viscoelasticity (Balazs and Denlinger, 1993).

Mechanism of action

The mechanism of HA action is not yet fully elucidated. HA reduces the sensitivity of articular nerve endings in the joint capsule by buffering transmission of mechanical forces to nociceptor nerve endings, thereby reducing pain (Gomis et al., 2004). HA also reduces

impulse activity in the peripheral nociceptor endings by affecting receptor channels (Caires et al., 2015).

Hyaluronan molecules restrict large plasma proteins from entering the SF, but facilitate the passage of small molecules to maintain nutrition (Goldberg and Buckwalter, 2005). It is believed that HA exogenously injected into the joint stimulates the synthesis of HA by synoviocytes and promotes proteoglycan synthesis by chondrocytes (Frean et al., 1999; Bagga et al., 2006), and thus, HA can also indirectly contribute to the viscosity of the SF and lubricate joints.

Furthermore, HA has an anti-inflammatory effect (Punzi et al., 1989; Frean and Lees, 2000; Gomis et al., 2004; Frisbie et al., 2009). HA attenuates inflammation by competitive inhibition, inhibition of degradative enzymes for collagen, aggrecan, and proteoglycan, and upregulation of proteoglycans, GAGs, TGF- β , and type II collagen (Lindholm et al., 2002; Santangelo et al., 2007; Legendre et al., 2008). *In vitro*, HA has been shown to decrease apoptosis and inflammatory mediator production of synovial cells (Kilborne et al., 2017). Furthermore, HA has been shown to block some of the detrimental effects in chondrocyte morphology exerted by LPS in equine articular cartilage *in vitro* (Bolt et al., 2008).

The half-life of unmodified HA solutions can be less than one day. Since the pain-relieving effect of IA HA within the injected joint often persists for considerably longer than the half-life of HA, it is likely that it provides not only symptom-modifying but also disease-modifying effects (Brown et al., 1991; Lindehayn et al., 1997).

Intra-articular use of hyaluronan

Exogenous IA HA has been shown to provide an analgesic effect and reduce pain in equine (Åsheim and Lindblad, 1976; Rose, 1979; Auer et al., 1980; Ruth and Swites, 1985; Aviad et al., 1988; Gaustad and Larsen, 1995) and human OA (Dougados et al., 1993; Åkermark et al., 2002; Altman et al., 2004; Balazs, 2004; Raman et al., 2008; Abate et al., 2010; Chevalier et al., 2010; Arden et al., 2014; Leighton et al., 2014; Vega et al., 2015; Zhang et al., 2015; Estades-Rubio et al., 2017).

A high MW non-animal stabilized hyaluronic acid (NASHA) product has gained popularity in the treatment of human arthritic conditions due to its long-acting pain-relieving effects (Åkermark et al., 2002; Goldberg and Buckwalter, 2005; Altman et al., 2004; Raman et al., 2008; Abate et al., 2010; Chevalier et al., 2010; Arden et al., 2014; Leighton et al., 2014; Vega et al., 2015; Zhang et al., 2015; Estades-Rubio et al., 2017). Following bacterial synthesis of HA, production of NASHA involves stabilization with molecular cross-linking. The resulting NASHA gel has an increased density and a long half-life of up to 32 days (Lindqvist et al., 2002; Edsman et al., 2011). NASHA remains in the synovial structures for a considerably longer period than other HA products (Lindqvist et al., 2002; Åkermark et al., 2002; Altman et al., 2004). NASHA is administered as a

single IA injection. It has been shown to provide significant benefits in the treatment of human knee OA, lasting for at least 6 months (Altman et al., 2004; Leighton et al., 2014).

IA HA injection is a commonly used treatment for equine synovitis and OA, although only a few clinical studies have been conducted on IA HA treatment without any concurrent medication in horses (Rose, 1979; Auer et al., 1980; Aviad et al., 1988; Ruth and Swites, 1985; Gaustad and Larsen, 1995). Previously, only one double-blinded placebo-controlled clinical equine study on IA HA treatment has been published (Gaustad and Larsen, 1995). This study reported a superior effect of combined HA and PSGAG groups compared with placebo in reducing the lameness score. In addition to clinical studies, the efficacy of IA HA in relieving inflammation and pain has only been examined in a few experimental equine studies (White et al., 1999; Frisbie et al., 2009a). In horses with medically induced synovitis, White et al. (1999) found HA to relieve lameness compared with placebo. However, in a surgically induced equine OA model, no changes in the lameness scores were detected after HA treatment compared with control treatment (Frisbie et al., 2009a).

Some OA models in animals have shown that high MW HA preparations are superior to less polymerized HA forms in prohibiting cartilage degeneration (Kikuchi et al., 1996; Shimizu et al., 1998), and in alleviating pain (Gotoh et al., 1993). Furthermore, high MW HA has been reported to stimulate endogenous HA production more than low MW formulations (Smith et al., 1987). In an early clinical equine study comparing HA preparations of different MW, better results in relieving lameness were described using a HA preparation with high MW (Phillips, 1989). However, more research on the different MW preparations is needed.

Adverse effects of hyaluronan

A transient treatment-related adverse effect of IA HA has been reported in up to 10.0% of cases in an equine study (Ruth and Swites, 1985) and up to 12.5% in a study of human OA treated with IA NASHA (Altman et al., 2004). Especially repeated IA HA injections have been found to induce a transitional IA inflammation reaction first (Leopold et al., 2002; Pullman-Mooar et al., 2002; Marino et al., 2006), likely caused by low MW breakdown products of the HA (Pullman-Mooar et al., 2002).

All reported NASHA treatment-related adverse effects have been local and mostly mild (Åkermarck et al., 2002; Altman et al., 2004; Vaquerizo et al., 2013; Arden et al., 2014; Leighton et al., 2014; Zhang et al., 2015). Since NASHA is produced entirely in a laboratory environment using non-animal sources, the risk of contamination with allergens or infectious agents of animal origin is minimal. No immune reactions have been described with NASHA. The most common adverse effect of NASHA in human joints is arthralgia (Altman et al., 2004). In contrast, after animal-derived HA, a variety of adverse effects have been reported, including local inflammation at the injection site, anaphylactic

reactions, pseudoseptic reactions, and granuloma formation (Leopold et al., 2002; Hamburger et al., 2003; Sasaki et al., 2003).

2.2.2 Corticosteroids, polysulphated glycosaminoglycans, and autologous conditioned serum

Intra-articular corticosteroids have been used since the 1960s (McIlwraith, 2010), and they are potent in attenuating IA signs of inflammation and lameness (Foland et al., 1994, Frisbie et al., 1997; Frisbie et al., 1998). However, the duration of the effect is rather brief; in treatment of human knee OA, at 4–24 weeks' post-injection, only little evidence of an effect has been reported (Bellamy et al., 2006). In addition, corticosteroids are potentially harmful to the articular cartilage. In equine studies, variation of deleterious effects has been shown between the different corticosteroids (Foland et al., 1994, Frisbie et al., 1997; Frisbie et al., 1998). The most commonly used IA corticosteroids in horses are betamethasone, triamcinolone, and methylprednisolone (McIlwraith, 2010). With bethamethasone, no deleterious side-effects on articular cartilage were demonstrated in an experimental equine study using the osteochondral fragment–exercise model. Furthermore, in that study, exercise was shown not to produce harmful effects in the presence of betamethasone (Foland et al., 1994). Similarly, triamcinolone seems to be safe or even beneficial for the articular cartilage (Frisbie et al., 1998), but after IA administration of methylprednisolone, deleterious histopathological changes were demonstrated in equine joints in experimental settings (Frisbie et al., 1997). Studies on human clinical OA have shown that with higher doses and longer treatments IA corticosteroids are associated with chondrotoxicity (Wernecke et al., 2015).

Polysulphated glycosaminoglycans (PSGAGs) are considered DMOADs, traditionally aimed at preventing or slowing down morphological lesions in the cartilage. The principal glycosaminoglycan (GAG) in PSGAGs, chondroitin sulphate (CS), is made from an extract of bovine lung and trachea modified by sulphate esterification (McIlwraith, 2011b). PSGAG treatment has been shown to result in increased collagen and GAG synthesis *in vitro* in equine articular cartilage (Glade, 1990). However, another *in vitro* study found a dose-dependent inhibition of proteoglycan synthesis (Caron et al., 1991). One experimental equine study using the osteochondral fragment–exercise model has demonstrated significantly less synovial membrane vascularity and subintimal fibrosis with PSGAG treatment compared with controls (Frisbie et al., 2009a). *In vitro*, PSGAG treatment has been shown to inhibit PGE₂ production after LPS stimulation in cultured equine synoviocytes (Frean and Lees, 2000). A slightly increased risk of infection following IA injection of PSGAG relative to corticosteroids and HA has been reported (Gustafson et al., 1989).

Interleukin 1 (IL-1) is one of the major mediators at the top of the cascade for cartilage degradation in OA (Roman-Blas and Jimenez, 2006). Autologous conditioned serum (ACS) is aimed at concentrating IL-1ra and blocking the effects of IL-1. In the production

of concentrated IL-1ra, peripheral blood is drawn into a syringe containing glass beads treated with chromium sulphate to which blood monocytes and other adherent cells attach. The syringe with its contents is then incubated at 37°C for several hours during which platelets degranulate and mononuclear cells synthesize and secrete IL-1ra along with a variety of additional anti-inflammatory products without a significant increase of IL-1 β and TNF- α (Frisbie et al., 2007).

No controlled clinical studies in horses concerning the clinical efficacy of ACS have been published. In a double-blinded, placebo-controlled, experimental equine study using the IA osteochondral fragment–exercise model, ACS was shown to improve lameness score, resulting in less synovial hyperplasia and less gross fibrillation of cartilage and synovial haemorrhage than placebo-injected joints (Frisbie et al., 2007). The SF concentration of IL-1ra assessed by using a mouse anti-IL-1ra antibody was also demonstrated to increase after treatment with ACS (Frisbie et al., 2007). In a controlled clinical study, pain relief and improved shoulder function were reported over a 24-week period in human patients treated with IA ACS, compared with patients treated with glucocorticoids (Damjanov et al., 2018). Furthermore, the efficacy of IA ACS in the treatment of symptomatic OA of the knee has been demonstrated in man (Tassaraa et al., 2018).

2.3 Molecular mediators as biomarkers in intra-articular inflammation and cartilage metabolism

Inflammatory and degenerative activity inside the joint can be studied *in vivo* via analyses of SF biomarkers, which are molecular markers of inflammatory processes and tissue turnover. In OA, synovial macrophages play a major role in immune activation and cytokine production (Sokolove and Lepus, 2013). In addition, other cells, including chondrocytes, synoviocytes, and osteoblasts, synthesize inflammatory cytokines such as interleukin-1 (IL-1), interleukin-4 (IL-4), interleukin-9 (IL-9), interleukin-13 (IL-13), and tumour necrosis factor alpha (TNF- α) (Steenworden et al., 2007; Man and Mologhianu, 2014; Wojdasiewicz et al., 2014; Glyn-Jones et al., 2015; Kraus et al., 2015). Inflammatory cytokines are the main promoters of catabolic and destructive processes in articular tissues, which lead to the loss of metabolic homeostasis in joints. Dysregulation and imbalance in the metabolism between anti-inflammatory and inflammatory cytokines in the joint eventually lead to OA (Wojdasiewicz et al., 2014).

Inflammatory cytokines affect most of the cells in the joint. They act via intracellular pathways of signal transduction on the production of other cytokines and other inflammatory compounds and enzymes, such as collagenases or matrix metalloproteinases (MMPs) and proteinases of the ADAMTS family, by chondrocytes, osteoblasts, and synoviocytes (Sokolove and Lepus, 2013; Man and Mologhianu, 2014; Glyn-Jones et al., 2015; Kraus et al., 2015). As a result, excessive amounts of inflammatory mediators,

including prostaglandin E₂ (PGE₂), cyclo-oxygenase-2, phospholipase A₂, nitrous oxide (NO), and free radicals, among other compounds, are produced. Moreover, inflammatory cytokines enhance their own production via autocrine and paracrine action (Wojdasiewicz et al., 2014). Inflammatory mediators attract immune cells, increase synovial angiogenesis, and induce chondrocytes to produce additional cytokines and proteolytic enzymes that further increase cartilage matrix degradation (Lindblad and Hedfors, 1987). Initially, aggrecan is fragmented and released from ECM of cartilage in OA, followed by other molecules such as cartilage oligomeric matrix protein (COMP), fibromodulin, and collagens. Molecules from degraded cartilage released into the synovial cavity may act as a foreign material in the SF and enhance synovial inflammation in OA. Some ECM proteins or their degradation products activate the complement pathways, consequently promoting joint inflammation. Further, increased permeability of blood vessels and cellular release of lysosomal enzymes ultimately damage the articular cartilage and lead to a sustained synovial inflammation (Silawal et al., 2018). In addition to the destructive impact on articular cartilage, inflammatory cytokines induce ageing and apoptosis of chondrocytes and decrease the synthesis of the key components of the extracellular ECM, such as proteoglycans, aggrecan, and type II collagen (Wojdasiewicz et al., 2014). Moreover, the nerve endings of the primary afferent neurons release neuropeptides into the joint, promoting inflammation and affecting chondrocyte function (Sutton et al., 2009).

Research into the biomarkers of equine SF has focused on finding tools for early diagnosis of OA and monitoring progression of the disease (Gibson et al., 1996; Todhunter et al., 1996; Kirker-Head et al., 2000; Bertone et al., 2001; Frisbie et al., 2008; Nicholson et al., 2010). Furthermore, equine SF biomarkers have been used to measure responses to treatments of synovitis and induced OA (van Loon et al., 2010; Frisbie et al., 2013) and to evaluate possible deleterious effects of IA medications (Robion et al., 2001; Piat et al., 2012) and autologous platelet-rich plasma (PRP) treatment (Moraes et al., 2015). Recently, protein profiles of equine SF by means of proteomic methods have been used to differentiate arthritic conditions in horses instead of measuring a predefined selection of SF biomarkers (Chiaradia et al., 2012; Anderson et al., 2019). The following sections introduce the inflammatory mediators, markers of cartilage metabolism, anti-inflammatory mediators, and anabolic growth factors explored in this study.

2.3.1 Inflammatory mediators

Prostaglandin E₂

Eicosanoid PGE₂ is one of the most important mediators in joint diseases. Elevated SF PGE₂ concentration along with increased lameness in horses have been shown in several experimental studies after induction of synovitis or OA (Owens et al., 1996; Kawcak et al., 1997; Schleining et al., 2008; Frisbie et al., 2009a; Frisbie et al., 2009b; de Grauw et al., 2009a; de Grauw et al., 2009b; van Loon et al., 2010; Frisbie et al., 2013). SF PGE₂

concentration has been considered applicable as a screening test for the presence of joint disease (Gibson et al., 1996; Kirker-Head et al. 2000; Bertone et al., 2001; Lucia et al. 2013), although high variability of the SF concentrations in the diseased joints limits its usefulness as a discriminator among the different types of joint diseases (Bertone et al., 2001). However, increase of SF PGE₂ concentration has been documented also in cases of unresponsive IA analgesia (de Grauw et al., 2006a). This indicates that either PGE₂ is a non-specific indicator of IA pain or the negative response to IA analgesia might not always exclude the painful condition within the joint cavity (de Grauw et al., 2006a). Furthermore, repeated arthrocentesis and exercise can cause a rise in PGE₂ concentration in equine SF (van den Boom et al., 2004).

The rise in the PGE₂ concentration is very rapid, peaking 2-9 h after the experimental induction (Owens et al., 1996; de Grauw et al., 2009a), but a long duration – up to 72 days – of increased concentration has been reported (Kawcak et al., 1997; Frisbie et al., 2008).

Substance P

Substance P is a pro-nociceptive neuropeptide that resides in small-diameter primary afferent neurons. Stimulation of these neurons leads to the release of substance P, which initiates a local inflammatory response (Marshall et al., 1990). Substance P enhances the production of prostaglandins and collagenases by synoviocytes (Lotz et al., 1987). Furthermore, it can activate macrophages, B-lymphocytes, polymorphonuclear cells, platelets, and mast cells (Menkes et al., 1993).

The concentration of substance P has been shown to increase in osteoarthritic equine (Kirker-Head et al., 2000) and human joints (Marshall et al., 1990). Interestingly, the density of substance P-secreting nerve endings in the synovial membrane is greater in the more distal joints of the appendicular skeleton than in the more proximal joints. This may account for the greater frequency of OA in the more distal joints of the horse (Caron et al., 1992). A relationship between elevated SF substance P concentration and clinical IA pain and lameness in horses has been reported (de Grauw et al., 2006a). However, in the experimental study using a lipopolysaccharide (LPS) induction model, an increase of SF substance P concentration for only 24 h was documented (de Grauw et al., 2009a).

Tumour necrosis factor alpha

Tumour necrosis factor alpha (TNF- α) is synthesized by chondrocytes, osteoblasts, cells of the synovial membrane, and mononuclear cells present in the joint or infiltrating there during the inflammatory process. TNF- α is a catabolic cytokine that affects chondrocytes by blocking the synthesis of proteoglycan components, protein binding proteoglycans, and type II collagen (Saklatvala, 1986).

TNF- α production in the joint is easily induced by both joint disease (di Giovine et al.,

1988; Ley et al., 2007; Kamm et al., 2010) and other types of stress such as physical exercise (Billinghurst et al., 1995; van den Boom et al. 2004; Rossetti et al., 2012). It was shown to relate to the long duration of OA in humans (di Giovine et al., 1988), but not to be useful as a biomarker of the type of disease in human or equine joints (di Giovine et al., 1988; Ley et al., 2007).

2.3.2 Markers of cartilage metabolism

Aggrecan chondroitin sulphate 846 epitope

Most of the extracellular matrix of articular cartilage is composed of proteoglycan aggrecan and type II collagen. Synthesis of aggrecan can be studied by analysis of the aggrecan chondroitin sulphate 846 epitope (CS846) (Lavery et al., 2000; Piat et al., 2012; Koenig et al., 2014). Inflammation induced with LPS has been reported to immediately increase the release of CS846 into the SF (de Grauw et al., 2009a). Long-standing release has been described also after surgically induced OA in the equine osteochondral fragment model (Koenig et al., 2014). However, lameness and IA pain are not necessarily related to increased cartilage matrix turnover and increased SF CS846 concentration (de Grauw et al., 2006a). A reduction of SF CS846 concentration has been reported in equine osteochondrosis and is suggestive of impaired aggrecan synthesis (Lavery et al., 2000).

Among the common IA treatments in horses, IA methylprednisolone has been shown to increase SF CS846 concentration. It was concluded to indicate, together with the increased keratan sulphate epitope, loss of articular cartilage proteoglycans (Robion et al., 2001). Similarly, a cartilage insult after IA bupivacaine and lidocaine has been suspected based on induction of CS846 release following the IA injection of these local anaesthetics (Piat et al., 2012).

Carboxypeptide of type II collagen

The metabolism of the other major component of the ECM, type II collagen, has been examined in several equine studies (Lavery et al., 2000; de Grauw et al., 2006a; Frisbie et al., 2008; Kawcak et al., 2008; de Grauw et al., 2009a; Nicholson et al., 2010). Carboxypeptide of type II collagen (CPII) is the marker of collagen II synthesis. It has been shown to increase for a prolonged period of up to 7 days in equine SF in the LPS-induced synovitis model (de Grauw et al., 2009a) and for even a more extended time (91 days) in SF of horses with surgically induced OA (Frisbie et al., 2008).

CPII concentration increases in SF in equine osteochondrosis (Lavery et al., 2000). It has been shown that an increase of SF CPII concentration is affected by the type of joint in naturally occurring osteochondral injury (Nicholson et al., 2010). However, as stated previously with CS846, clinically detected intra-articular pain is not always related to

changes in cartilage turnover and change in CII (de Grauw et al., 2006a). Therefore, this direct biomarker, as other markers of cartilage metabolism alone, may not be a reliable indicator of joint pathology (de Grauw et al., 2006a). IA injection of local anaesthetics has been reported to cause an increase in concentration of CII in equine SF, suggesting a reparative response after undetected cartilage injury (Piat et al., 2012). On the contrary, a reduction of SF CII concentration has been documented after IA methylprednisolone, which was speculated to result from inhibition of collagen synthesis (Robion et al., 2001).

2.3.3 Interleukin-1 receptor antagonist

Interleukin-1 receptor antagonist (IL-1ra) is produced by several cell types, including monocytes, synoviocytes, and chondrocytes (Arend et al., 1990; Smith et al., 1991). IL-1ra blocks the interleukin-1 (IL-1) receptors, and thus, inhibits IL-1 binding and activity (Muller-Ladner, 1997). The binding of IL-1 β to the receptor results in activation of several transcription factors and expression of hundreds of genes whose products include other cytokines, chemokines, adhesion molecules, inflammatory mediators, and enzymes (Roman-Blas and Jimenez, 2006). By blocking the IL-1 receptor, significant effects of IL-1 on the metabolism of cells and ECM and also on IL-1-mediated degradation of cartilage is prohibited (Muller-Ladner, 1997). Furthermore, IL-1ra has been shown to decrease intimal hyperplasia in equine joints and, on a large scale, to decrease pain (Caron et al., 1996). Decreased SF IL-1ra concentration has been demonstrated in the chronic stages of human OA (Bigoni et al., 2017).

2.3.4 Anabolic growth factors

Transforming growth factor beta 1

Transforming growth factor beta 1 (TGF- β_1) is an essential protein in anabolic events in OA, and it diminishes joint pain and inflammation (Fortier et al., 2011). It is stored mainly in the alpha granules of platelets. TGF- β_1 is capable of inducing chondrogenic differentiation of mesenchymal stem cells with rapid biosynthesis of glycosaminoglycan and deposition of an integrated ECM (Barry et al., 2001; Tuli et al., 2003). In addition, TGF- β_1 activates multiple pathways responsible for cell survival, growth, and metabolic regulation (Zhai et al., 2015). However, increased expression of TGF- β_1 has been demonstrated with developing osteophytes (Scharstuhl et al., 2002) and hyperplasia of the synovium (Backer et al., 2001). Possibly only a narrow concentration range of bioactive TGF- β is beneficial for cartilage health, and any concentration below or above this range may cause aberrant alterations in TGF- β pathways and lead to abnormal cartilage function (Finnson et al., 2012).

In humans, SF TGF- β concentration has been documented to be low or even undetectable in OA subjects (Blaney et al., 2006). Inflammatory cytokines have been suggested to intercept cell signalling pathways and result in the reduced production of TGF- β in the course of OA (Derynck and Zhang, 2003). TGF- β is an essential growth factor supporting aggrecan synthesis, and reduced mRNA expression of TGF- β has been observed in early lesions of equine osteochondrosis (Lillich et al., 1997).

Platelet-derived growth factor-BB

Platelet-derived growth factor BB (PDGF-BB) is one of the earliest and most sensitive growth factors expressed after tissue injury (Girolamo et al., 2015). It acts locally, is produced by smooth muscle cells, fibroblasts, endothelial cells, and macrophages, and is stored primarily by platelets (Lee, 2000). Platelet-derived growth factor (PDGF) induces proliferation and differentiation of fibroblasts, deposition of collagen, and angiogenesis (Mustoe et al., 1991; Wu et al., 1997). In addition, since it induces the synthesis of other growth factors (Creaney and Hamilton, 2008), it is an essential promoter of the healing process. Furthermore, the presence of PDGF-BB in cartilage defects exerts chemotactic and mitogenic effects and could stimulate the infiltration of mesenchymal stem cells onto the site (Liebesny et al., 2016). It also increases chondrocyte proliferation, differentiation, and cartilage proteoglycan production (Kieswetter et al., 1997).

PDGF-BB down-regulates the most important pro-inflammatory cytokines, IL-1 and TNF- α , and thus, has an anti-apoptotic effect on chondrocytes (Montaseri et al., 2011). SF PDGF-BB concentration of healthy horses has been measured only in two studies *in vivo* (Textor et al., 2013; Ríos et al. 2015a), but PDGF-BB concentrations in inflamed equine joints have not been reported.

3 Aims of the thesis

This work originates from studies in humans reporting promising effects of IA HA on joint pain relief in osteoarthritic patients and on SF inflammatory mediators and catabolic mediators related to inflammation. We hypothesized that IA HA (NASHA) would produce significant improvement in lameness and other pain-related clinical signs in horses. We also investigated the short-term mechanism of the anti-inflammatory action of NASHA inside the joint.

Detailed aims and hypotheses of the studies were as follows:

1. To examine the efficacy of IA NASHA in the treatment of lameness and related clinical signs of IA inflammation (effusion score, flexion test score, and flexion pain score) originating from a metacarpophalangeal joint (I) and an intercarpal joint (III). The hypothesis was that the lameness grade and clinical signs would improve more in horses treated with NASHA than in those treated with placebo (I) or receiving no treatment (III).
2. To investigate SF PGE₂, substance P, CS846, and CPII concentrations before and two weeks after IA NASHA or placebo injections in horses suffering clinically from pain in metacarpophalangeal joints (II). The hypothesis was that the decrease in SF biomarker concentrations would be greater in the NASHA group than in the placebo group.
3. To compare SF anti-inflammatory mediator IL-1ra, anabolic growth factors PDGF-BB and TGF- β_1 , and pro-inflammatory cytokine TNF- α concentrations before and after surgically induced cartilage defect in the intercarpal joint and after IA NASHA injection in horses (III). The hypothesis was that the concentrations of anti-inflammatory mediators, anabolic growth factors, and the concentration of TNF- α increase more in the SF of cartilage defect joints than in the sham-operated joints. Furthermore, we hypothesized that the concentrations of anti-inflammatory cytokine IL-1ra and anabolic growth factors PDGF-BB and TGF- β_1 will increase and the concentration of TNF- α will decrease more in the NASHA-treated joints than in the non-treated joints.
4. To monitor adverse effects of the IA NASHA injection on clinical signs (I,III) and SF parameters (II,III). The hypothesis was that IA NASHA would be well tolerated without exacerbated signs of joint inflammation, such as effusion, and that the WBC count and the concentrations of markers of inflammation would not increase in SF after the NASHA injection compared with the control group.

4 Materials and methods

4.1 Approval of study protocols

The clinical study was approved by the Viikki Campus Research Ethics Committee of the University of Helsinki (31 March 2010). Every horse owner signed a consent form before the start of the study, and the owner was able to rescind consent without any particular reason. The protocol of the experimental study was approved by the National Animal Experimental Board in Finland (ESA VI/1130/04.10.03/2011).

4.2 Study populations

4.2.1 Clinical patients (I,II)

The study was advertised at stables, on the internet page of the Faculty of Veterinary Medicine, in equine clinics, and in Finnish horse sport magazines. Horse owners contacted the Veterinary Teaching Hospital to have their horses included in the study and a telephone interview was conducted. An assistant not otherwise participating in the study enrolled all participants. Horses suffering lameness and IA inflammation of the metacarpophalangeal or metatarsophalangeal joint of various duration were eligible. Only adult, non-geriatric (i.e. aged 4–17 years) Finnhorse, Standardbred, and Warmblood horses were eligible. In addition, large-sized ponies (withers 140–148 cm) were accepted (they are referred to as horses in the text). Inclusion criteria were as follows: a positive response to diagnostic intra-articular analgesia and no significant radiographic signs (grade 0 or grade 1) of remodelling of the affected joint. This excluded horses with more advanced OA. In addition, horses with IA osteochondral or other fragments documented in radiographs were excluded. Bilaterally lame horses and horses that had received IA medications, such as corticosteroids, HA, or other medication within the previous three months, or peroral non-steroidal anti-inflammatory drugs (NSAIDs) within 15 days, were not eligible. Furthermore, horses with concurrent pathologies, such as clinically significant ligament, tendon, or other soft tissue injuries, in the affected limb were excluded.

Sixty-eight horse owners were interviewed and 36 horses were invited to the first clinical baseline examination. Altogether, 30 horses fulfilling the inclusion criteria were included in the study. However, as only three horses that finished the study had lameness due to synovitis of the metatarsophalangeal joint, these were omitted from some of the statistical analyses of clinical outcome variables. This was also done to prevent bias, as the detection and scoring of hindlimb lameness is more variable and subjective than that of

the front limb (Keegan et al., 2010), and hindlimb flexion tests are less specific than forelimb tests because the reciprocal apparatus prevents flexion of any joint without concomitant flexion of other joints (Ross, 2011). As a result, 27 horses with synovitis of the metacarpophalangeal joint were left as the primary study population. All the 27 horses completed the study (I). For the biomarker analyses (II), two out of 30 horses were excluded due to an inadequate amount of high-quality SF sample for the baseline and control investigations; i.e. 28 horses were included in the SF studies (Figure 1, Table 2).

Table 1. Clinical history of horses in the clinical study (I,II).

	n (%)
Amount of exercise daily	
0.5-1 hour	21 (70%)
>1 hour	9 (30%)
Level of exercise daily	
Light	11 (36.7%)
Moderate	18 (60%)
Vigorous	1 (3.3%)
Mean exercise, days/ week	5.1
Mean exercise, hours/week	6.2
Previous lameness of affected limb	24 (80%)
Previous lameness localized to affected joint	18 (60%)
Previous treatment for orthopaedic problems:	
IA corticosteroids	11 (36.7%)
IA hyaluronan	1 (3.3%)
Other IA treatment	6 (20%)
NSAIDs perorally	2 (6.7%)
No previous treatments	9 (30%)
Not known	1 (3.3%)
Duration of lameness/ poor performance	
< 1 month	3 (10%)
1-2 months	7 (23.3%)
>2-4 months	5 (16.7%)
>4-12 months	4 (13.3%)
>12 months	9 (30%)
Not known	2 (6.7%)

n=number of horses; IA, intra-articular; NSAIDs, non-steroidal anti-inflammatory drugs

Table 2. Number of participating horses, signalment, and number of horses with findings in radiographs in the clinical study (I,II).

Variable	Placebo	HA I/II
Number of horses	13	14/15
Age (years)		
Mean (range)	8.4 (4-17)	7.2 (4-12)
Gender		
Mare	6 (46.2%)	6/7(42.9/46.7%)
Stallion	2 (15.4 %)	3 (21.4/20.0%)
Gelding	5 (38.5%)	5 (35.7/33.3%)
Purpose		
Harness race horse	7 (53.8%)	10 (71.4/66.7%)
Riding horse	6 (46.2%)	4/5(28.6/33.3%)
Breed		
Standardbred	4 (30.8 %)	6 (42.9/40.0%)
Finnhorse	5 (38.5%)	7/8(50.1/53.3%)
Warmblood	2 (15.4%)	1 (7.1/6.7%)
Pony	2 (15.4%)	0
Grade 1 findings in radiographs		
Yes	6 (46.2%)	7 (50.0/46.7%)
No	7 (53.8%)	7 (50.0/53.3%)

4.2.2 Experimental horses (III)

Eight Standardbred horses (four mares, one stallion, and three geldings) free of lameness and pathological findings in intercarpal joints were used in the experimental study. The horses were donated to the University of Helsinki for this study. The horses were to be euthanized afterwards. A person not participating in the study recruited the horses. All horses used for the study fulfilled the inclusion criteria of general and orthopaedic health, i.e. horses were considered healthy based on clinical examination, routine haematology, and serum biochemistry analyses and were free of front limb lameness. Furthermore, horses were free of OA of intercarpal joints, as five radiographic views (dorsopalmar,

dorsolateral-palmaromedial, dorsomedial-palmarolateral, flexed lateromedial, and flexed dorsoproximal-dorsodistal) of the carpal joints were assessed, and IA surfaces of intercarpal joints were inspected by bilateral arthroscopic surgery to ensure that no visible pre-existing abnormalities were present in the joints.

4.3 Study design

4.3.1 Clinical study (I,II)

The clinical study was carried out as a randomized, double-blinded, and placebo-controlled trial with a parallel group design and equal allocation ratio. It was designed and reported according to the Consolidated Standard of Reporting Trials (CONSORT) statement.

Preplanned criteria for removing a horse from the study were side-effects caused by the NASHA or placebo products, additional orthopaedic or other health problems occurring after the first clinical examination, and/or the owner's lack of compliance or failure to follow instructions given after the first clinical examination. Before starting the study, a non-blinded assisting technician created a computer-generated randomization list using an internet-based program (Research randomizer, <https://www.randomizer.org/> (2010)). The block size was 4 and no strata were used. Horses fulfilling the inclusion criteria were assigned to the treatment or control group according to the randomization list. The list and the NASHA and placebo products were kept in a locked locker at the university that only the non-blinded assisting veterinarian and his technician could access. Treatments were double-blinded so that the assisting veterinarian prepared the syringes according to the randomization list and performed the NASHA and saline injections. Neither the evaluating veterinarian (author) nor the owners were allowed in the treatment room during the procedure. Owners remained blinded until after the second clinical examination and the evaluating veterinarian (author) until after the statistical analyses were performed.

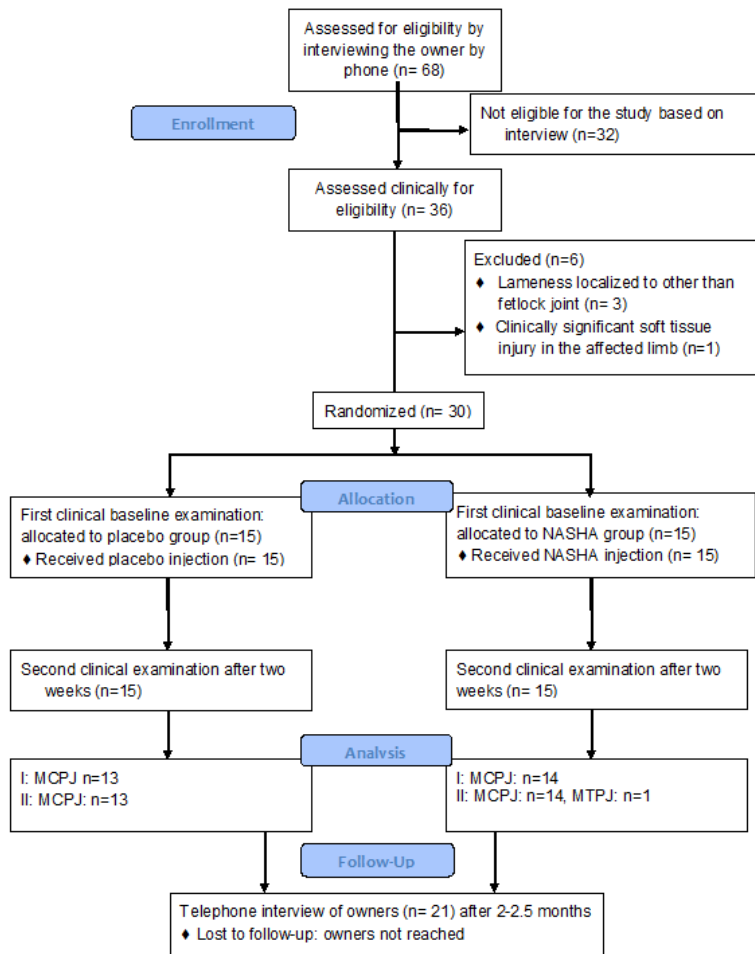


Figure 1. *Flow chart showing the number of horses in different phases of the clinical study. MCPJ, metacarpophalangeal joint; MTPJ, metatarsophalangeal joint*

4.3.2 Experimental study (III)

In this study, the contralateral intercarpal joint served as a control for both interventions. Before recruiting the horses, a combination of interventions was randomly designated for each right intercarpal joint. As a result, each joint was selected for one of the following: Cartilage defect (CD) with NASHA (CD+NASHA+), CD without NASHA (CD+NASHA-), Sham-operation (SO) with NASHA (SO+NASHA+), or SO without NASHA (SO+NASHA-). Interventions for each of the contralateral intercarpal joints were

determined by these randomly designated combinations; i.e. if CD with NASHA was selected for the right side, the left side was done as SO without NASHA injection (Table 3). At day 0, a CD was surgically created for one of the intercarpal joints of each of the eight horses, while the other joint served as a sham-operated control. At day 5, 3 ml (20 mg/ml) of IA NASHA (Durolane, Bioventus LLC, Durham, NC, USA) was injected into one of the intercarpal joints so that four of the CD joints were injected and four of the SO joints were injected, as described. The contralateral intercarpal joint served as the non-treated control (Table 4).

4.4 Examination procedures

4.4.1 Clinical study: baseline clinical examination (I,II)

Within a week after the horse owner had contacted the Veterinary Teaching Hospital to have their horse considered for inclusion, the horse was presented to the Veterinary Teaching Hospital of the University of Helsinki for the first baseline clinical examination (Figure 1). The examination was performed on the same day that the horse arrived at the hospital.

Lameness examination

The horses were subjected to a complete lameness examination by the evaluating veterinarian (author). A standardized American Association of Veterinary Practitioners (AAEP) scale of 0–5 (0 = lameness not perceptible under any circumstances, 1 = lameness is difficult to observe and is not consistently apparent, regardless of circumstances, 2 = lameness is difficult to observe at a walk or when trotting in a straight line but consistently apparent under certain circumstances (e.g. weight-carrying, circling, inclines, hard surface, etc.), 3 = lameness is consistently observable at a trot under all circumstances, 4 = lameness is obvious at a walk, 5 = lameness produces minimal weight bearing in motion and/or at rest or a complete inability to move; Anonymous, 1991) was used to grade lameness. Effusion of the affected joint was recorded on a scale of 0–4 (0 = no effusion, 1 = mild, 2 = moderate, 3 = severe effusion, 4 = severe swelling of the joint region) (White et al., 1999; de Grauw et al., 2009a). Other palpation findings, such as thickening of the joint capsule, were also recorded (yes/no).

Pain when flexing the affected distal limb was recorded on a scale of 0–3. This pain score was created by the author and was recorded as follows: 0 = no pain on flexion, 1 = mild pain, i.e. the horse shows some reaction, such as moving the limb, 2 = moderate pain, i.e. the horse retracts the limb repeatedly during the 1-min flexion period, 3 = severe

pain, i.e. the flexion test cannot be properly performed. A flexion test of the affected and the contralateral distal limb was performed, and the change in lameness was recorded on a scale of 0–4 (0 = no increase, 1 = slight increase, 2 = moderate increase, 3 = considerable increase in baseline lameness, 4 = non-weight-bearing lameness) (Frisbie et al., 2009a). To exclude confounding flexion reactions due to, for instance, sensitivity to handling and to better evaluate the reaction of the affected limb, the contralateral non-lame limb was always flexed first.

Synovial fluid sampling and intra-articular analgesia

Five millilitres of SF was aspirated into a sterile 5-ml syringe for the biomarker measurements. Arthrocentesis was performed with an 18-gauge/3.8-cm needle through the lateral sesamoidean ligament, and visible blood contamination in the SF sample was recorded. The SF sample was immediately divided into a plain tube on ice (4 ml) and an ethylenediaminetetraacetic acid (EDTA) (1 ml) tube. White blood cell (WBC) count and total protein (TP) concentration measurements were done from the fresh sample in the EDTA tube.

To localize the lameness and to decide whether the horse was eligible for the study, a routine diagnostic IA analgesia was performed after synoviocentesis by injecting 10 ml of mepivacaine hydrochloride (Scandicain, Astra Zeneca, Cambridge, UK) into the joint. The response to the IA analgesia was evaluated at 10 min post-injection and was considered positive if subjectively evaluated 80–100% amelioration of lameness was evident.

Radiographic examination

Radiographic examination of the joint with four standard views was performed after the lameness examination (lateromedial, dorsopalmar, dorsolateral-palmaromedial, and dorsomedial-palmarolateral). All radiographs were evaluated by the same veterinarian (author). For grading of changes in radiographs, the scale 1-4 was used (Robert et al., 2006), and only no findings or grade 1 changes (radiographic finding usually without clinical significance, e.g. mild modelling) were eligible for the study.

Intra-articular hyaluronan and placebo injections

Horses in the treatment group were injected with 3 ml (20 mg/ml) NASHA into the affected joint, while horses in the control group received an equivalent volume of the sterile 0.9% saline solution (Sodium Chloride 9 mg/ml, B Braun, Melsungen, Germany) into the joint (Table 3). Injections were administered on the same day that the horse

arrived at the hospital, after confirming eligibility and the source of lameness and evaluating the radiographs.

Table 3. Study design, procedures, and laboratory analyses in the clinical (I,II) and experimental study (III).

	I	II	III
Number of horses	n = 27	n = 28	n = 8 (16 joints)
Breed of horses	Multiple	Multiple	Standardbred
Type of the study	Clinical double-blind Placebo-controlled	Clinical double-blind Placebo-controlled	Experimental controlled
Investigated joint	MCPJ	MCPJ/MTPJ	Intercarpal
Intervention(s)	IA NASHA	IA NASHA	1. Surgical induction of CD 2. IA NASHA
Horses or joints/group	n = 14	n = 15	4 CD+NASHA+ 4 CD+NASHA- 4 SO+NASHA+ 4 SO+NASHA-
Control	IA placebo (saline) n = 13	IA placebo (saline) n = 13	1. Sham operation 2. No treatment
Lameness examination	2	2	3
Radiographic examination	1	1	1
SF laboratory analyses:			
WBC count		2	3
TP		2	3
PGE ₂		2 x duplicate	
Substance P		2 x duplicate	
CS846		2 x triplicate	
CPII		2 x duplicate	
IL-1ra			3 x triplicate
PDGF-BB			3 x triplicate
TGF- β_1			3 x triplicate
TNF- α			3 x triplicate

MCPJ, metacarpophalangeal joint; MTPJ, metatarsophalangeal joint; IA, intra-articular; NASHA, non-animal stabilized hyaluronic acid; CD+NASHA+, cartilage defect with hyaluronan; CD+NASHA-, cartilage defect without hyaluronan; SO+NASHA+, sham operation with hyaluronan; SO+NASHA-, sham operation without hyaluronan; WBC, white blood cell; TP, total protein; PGE₂, prostaglandin E₂; CS846, aggrecan chondroitin sulphate 846 epitope; CPII, carboxypeptide of type II collagen; IL-1ra, interleukin-1 receptor antagonist; PDGF-BB platelet-derived growth factor BB; TGF- β_1 , transforming growth factor beta-1; TNF- α , tumour necrosis factor alpha

4.4.2 Clinical study: second clinical examination (I,II)

Lameness examination

Starting from the following day after the IA injection, the horses were allowed 30 min hand-walking per day and free access to a small paddock. After two weeks, the second clinical examination was performed. Lameness grade, effusion grade, pain in flexion, and flexion test grade were re-evaluated and recorded (TMN).

Synovial fluid sampling and hyaluronan injection

After the second clinical examination, the double-blinded trial part was concluded and the horses in the control group also received an IA NASHA injection to treat the synovitis (Figure 1). A sampling and clinical examination interval of two weeks was chosen to optimize both timing of sampling (not too early to avoid the potential effect of first arthrocentesis) and the clinical effect of NASHA, while simultaneously not withholding treatment and training of client-owned horses for an unnecessarily long period. To ensure that all treatment injections were performed in the same manner, the assisting veterinarian also injected the placebo group with the NASHA. Follow-up information was collected two and a half to three months post-treatment (NASHA) by interviewing all owners by telephone.

4.4.3 Experimental study: lameness examinations, surgical procedures, synovial fluid sampling, and IA injection (III)

Table 4. Schedule for procedures performed in the experimental study (III).

	Day 0	Day 5	Day 14
CD	n = 8		
SO	n = 8		
NASHA		n = 8 (CD: n = 4, SO: n = 4)	
No NASHA		n = 8 (CD: n = 4, SO: n = 4)	
Interventions	Lameness examination	Lameness examination	Lameness examination
	SF sampling	SF sampling	SF sampling
	Arthroscopy	NASHA IA	Euthanasia

CD, cartilage defect; SO, sham operation; NASHA, non-animal stabilized hyaluronic acid; SF, synovial fluid

Lameness and radiographic examinations

The horses were subjected to a complete lameness examination similar to that in the clinical study (I,II) by the evaluating veterinarian (author) (Section 4.4.1).

Pain when flexing the carpus (III) was recorded on a scale of 0–3. This pain score was created by the authors and was recorded as follows: 0 = no pain on flexion, 1 = mild pain, i.e. the horse shows some reaction, such as moving the limb, 2 = moderate pain, i.e. the horse retracts the limb repeatedly during the 1-min flexion period, 3 = severe pain, i.e. the flexion test cannot be properly performed.

Radiographic examination of the carpal joints with the following views was performed: dorsopalmar, dorsolateral-palmaromedial, dorsomedial-palmarolateral, flexed lateromedial, and flexed dorsoproximal-palmarodistal). All radiographs were evaluated and graded by the same veterinarian (author), as described for the clinical study (in Section 4.4.1). Horses with no findings in radiographs or grade 1 changes (radiographic finding usually without clinical significance, e.g. mild modelling) were eligible for the study.

Synovial fluid sampling

Before the surgical procedure, 5 ml of SF of both intercarpal joints of each horse was aspirated into a sterile 5 ml syringe for the biomarker measurements. The SF sample was immediately divided into a plain 4 ml tube on ice and an EDTA tube. White blood cell (WBC) count and total protein (TP) concentration measurements were done from the fresh sample in the EDTA tube.

Surgical procedures

Horses underwent bilateral arthroscopic surgery of intercarpal joints under general anaesthesia. Horses participated also in a simultaneously conducted anaesthesia study not reported on here. During the arthroscopic procedure a CD was created on the dorsal edge of the third carpal bone of one randomly selected intercarpal joint of each horse. The lesion was generated by use of a 5.5 mm x 13 cm Dyonic Arthroscopic Surgery Blade (Smith & Nephew Inc., Andover, MA, USA). After the procedure, the debris was not actively lavaged from the joint, thereby inducing synovitis. During the surgical procedure also synovial membrane and joint capsule tissue samples (approximately 3 mm x 3 mm) were harvested with the scalpel from the dorsal region of the joint and placed in neutral-buffered 10% formalin for 24 h for haematoxylin and eosin staining for a study to be reported elsewhere. The SO joint (control) was only evaluated by using an arthroscope,

and SF, synovial membrane, and joint capsule tissue samples were harvested, but the cartilage was left intact.

The arthroscopic portals were closed, the forelimbs were bandaged, and the horses were allowed to recover from the anaesthesia. They were housed in stall boxes during the two-week study period. The status of each horse, including comfort, lameness at walk, body temperature, heart rate, and respiratory rate, was checked three times daily.

Second intervention

Five days post-surgery, the lameness examination was repeated, second SF samples from both intercarpal joints were harvested, and one previously randomly selected intercarpal joint of each horse was injected with 3 ml of IA NASHA (20 mg/ml) (Table 4).

Third intervention

Nine days after the IA NASHA injection (i.e. two weeks after the surgical arthroscopic procedure), the third lameness evaluation, the third SF sampling, and the second synovial soft tissue sampling of both intercarpal joints were again performed under general anaesthesia due to the simultaneous anaesthesia study, after which horses were euthanized on the operating table by an intravenous injection of 50 ml of T61 vet (Intervet International GmbH, Feldstrasse, Unterschleissheim, Germany) (Table 4).

4.5 Laboratory analyses

Processing of samples and determination of SF WBC count and TP concentration (II,III)

Within 1 h of collection, the plain sample was centrifuged at 12000g for 10 min at 4°C, after which it was aliquoted and stored at -80°C. SF samples were digested with 0.5 mg/ml hyaluronidase from bovine testes for 30 min at 37°C prior to analyses of SF PGE₂, substance P, CS846, and CPII (II). WBC count was calculated manually by using the Bürker chamber. TP concentration was determined by using the Konelab Prime 60i (Fisher Scientific, Vantaa, Finland) (II,III).

Prostaglandin E₂ (II)

Prostaglandin E₂ (PGE₂) was measured using a commercial enzyme immunoassay kit (PGE₂ high sensitivity EIA kit, Enzo Life Sciences, Plymouth Meeting, PA, USA),

intended for use for any species. Sample extraction was performed according to the manufacturer's instructions prior to the immunoassay. Briefly, SF samples were acidified by the addition of 1 M hydrochloric acid to pH 3.5 and then vortexed and centrifuged for 2 min at 12000 g. The samples were applied to a C18 cartridge previously conditioned with ethanol and water, and then flushed with water, 15% ethanol, and finally hexane. PGE₂ was eluted from the cartridge with ethyl acetate and stored as an elution solution at -80°C until analysis. The extracted samples were analysed within one week. At the time of the assay, the samples were evaporated to dryness in a vacuum evaporator and reconstituted with assay buffer and analysed according to the manufacturer's instructions. The samples were extracted and analysed in duplicate. Parallelism was tested for dilutions between 1:2 and 1:50. An appropriate dilution was made for every sample. The detection range was 7.81 to 1000 pg/ml with a detection limit of 11 pg/ml. Recovery of the extraction was 81% to 114%. The intra-assay coefficient of variation (CV) was 14.6%.

Substance P (II)

Substance P was measured using a commercial enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA) according to the manufacturer's instructions. The assay included rabbit polyclonal capture antibody. Although antibody was raised against human substance P, the kit can be used for equine samples since the amino acid sequence of equine substance P is identical to that of human substance P (Studer et al., 1973). Samples were analysed in duplicate. The detection range was 3.9 to 500 pg/ml with a detection limit of 10.5 pg/ml. The intra-assay CV was 12.5%.

Aggrecan chondroitin sulphate 846 epitope (II)

Aggrecan chondroitin sulphate 846 epitope was measured using a commercial enzyme immunoassay kit (IBEX Pharmaceuticals Inc., Montreal, Quebec, Canada) according to the manufacturer's instructions. The assay is validated for use in the horse (Frisbie et al., 1999, de Grauw et al., 2006b). The samples were diluted 1:50 and added to plates in triplicate. The result was reported as the mean of triplicate values, unless the CV was over 20%; in this case, the mean of the two closest values was calculated. The detection range was 20 to 1000 ng/ml with a detection limit of 20 ng/ml. The intra-assay CV was <20%.

Carboxypeptide of type II collagen (II)

Carboxypeptide of type II collagen was measured using a commercial enzyme immunoassay kit (IBEX Pharmaceuticals Inc., Montreal, Quebec, Canada) according to the manufacturer's instructions. The assay is validated for use in the horse (Frisbie et al.,

1999, de Grauw et al., 2006b). Samples were diluted 1:5 and added to plates in duplicate. The detection range was 50 to 2000 ng/ml with a detection limit of 50 ng/ml. The intra-assay CV was 8.1%.

Interleukin 1 receptor antagonist (III)

Interleukin 1 receptor antagonist was measured using a commercial enzyme immunoassay kit (Equine IL-1ra/IL1F3 DuoSet, DY1814, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions and assayed with equine-specific antibodies. Samples were analysed in triplicate. The detection range was 312 to 20000 pg/ml with a detection limit of 312 pg/ml. The intra-assay CV was <6%.

Platelet-derived growth factor BB (III)

Platelet-derived growth factor BB was measured using a commercial enzyme immunoassay kit (Human PDGF-BB DuoSet, DY220, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions and using human antibodies, as high homology between these proteins in humans and horses exists (Penha-Goncalves et al., 1997). Samples were analysed in triplicate. The detection range was 31.25 to 2000 pg/ml with a detection limit of 31.25 pg/ml. The intra-assay CV was <6%.

Transforming growth factor beta 1 (III)

Transforming growth factor beta 1 was measured using a commercial enzyme immunoassay kit (Human TGF- β_1 DuoSet, DY240E, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions and using human antibodies, as high homology between these proteins in humans and horses exists (Donnelly et al., 2006). Samples were analysed in triplicate. The detection range was 31.25 to 2000 pg/ml with a detection limit of 31.25 pg/ml. The intra-assay CV was <6%.

Tumour necrosis factor alpha (III)

Tumour necrosis factor alpha was measured using a commercial enzyme immunoassay kit (Equine TNF-alpha DuoSet, DY1814, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions and using equine-specific antibodies. Samples were analysed in triplicate. The detection range was 31.25 to 2000 pg/ml with a detection limit of 31.25 pg/ml. The intra-assay CV was <6%.

4.6 Outcome measures

4.6.1 Clinical study (I,II)

The primary outcome measure of clinical signs was the change in the lameness score from baseline to the second clinical examination in the two intervention groups. The secondary outcome measures of clinical signs were the change in effusion of the affected joint, in lameness after the flexion test, and in degree of pain in flexion from baseline to the second clinical examination. The evaluated adverse effects were deteriorating of clinical signs in the second clinical examination and/or severe lameness and effusion of the treated joint acutely after the injection.

The outcome measure of laboratory analyses was the change in the SF PGE₂, substance P, CS846, and carboxypeptide CPII concentrations from baseline to the second SF sampling in the NASHA and placebo groups. The evaluated adverse effects were an increase in SF WBC count and an increase in SF PGE₂, substance P, and TP concentrations.

4.6.2 Experimental study (III)

The primary outcome measures were the change in SF IL-1ra, PDGF-BB, TGF- β_1 , and TNF- α concentrations and in clinical signs from baseline to the second SF sampling five days after the surgical intervention (induction of CD and SO) and nine days after the IA treatment (NASHA or no treatment) in four treatment groups. The secondary outcome measures were the change in SF IL-1ra, PDGF-BB, TGF- β_1 , and TNF- α concentrations after the surgical interventions in all joints in combined groups, i.e. regardless of the operation type. The evaluated adverse effects after the NASHA injection were worsening of clinical signs of IA inflammation and an increase in SF TNF- α concentration, WBC count, and TP concentration.

4.7 Statistical analyses

4.7.1 Clinical study (I,II)

A sample size calculator (Working in Epidemiology, 2019, <http://www.winepi.net/uk/index.htm>) was used with a 95% confidence level and 80% power, and the adequate sample size was estimated to be 11–14 horses per group based on two different percentages for the placebo group and 87% of cases showing clinical improvement in an earlier study on HA for the treatment of naturally occurring arthritic

conditions in horses (Ruth and Swites, 1985). In the placebo group, the proportion that would improve was estimated to be 20–30%, where 20% is 10% lower than the percentage that has been used in the placebo groups in human studies (Pham et al., 2004; Richette et al., 2009).

The final values for the clinical outcome measures were subtracted from the baseline values to form variables of change, allowing positive and negative values. These variables were used in the comparison between groups with the independent-samples Mann-Whitney U-test (ranks). The demographic data and outcome measures at baseline were compared between the two treatment groups using the independent-samples Mann-Whitney U-test, or in the case of nominal categorical variables, Fisher's exact test or the likelihood ratio test. Furthermore, the outcome measures within groups were compared between the baseline examination and the second clinical examination using the Wilcoxon signed-rank test. All of the above statistics were calculated for the population intended to treat ($n = 30$) and for the metacarpophalangeal patients only ($n = 27$). $P < 0.05$ was considered significant. Data analysis was performed with a computer-based statistical program (SPSS Software, IBM, New York, NY, USA) (I).

SF biomarker (PGE₂, substance P, CS846, CPII) concentrations, WBC count, and TP concentration measurements were examined with analysis of covariance models. Changes between baseline and follow-up measurements were used as the response, treatment as the fixed effect, and the corresponding baseline measurement as the covariate. For SF concentrations of substance P and CPII and SF WBC count, a logarithmic transformation was used to normalize the distributions. For PGE₂ concentration, an inverse transformation was used to normalize the distributions. CS846 and TP were normally distributed.

The possible effects of background variables (age, gender, breed, use of the horse, lameness score, duration of clinical signs, previous incidences of the affected joint, radiographic findings) on the change in the different biomarker concentration measurements from baseline were assessed with analysis of variance models. The models included the background variable of interest as the sole fixed effect and the change of the biomarker concentration from baseline as the response. If some of the background variables were statistically significant in these analyses, the effect was also included in the analysis of covariance model for the treatment comparison.

We hypothesized that the decrease of these SF biomarker concentrations would be greater in the NASHA group than in the placebo group. The difference in the change of each SF biomarker concentration after IA injection and a two-sided 95% confidence interval for the difference were estimated from the fitted analysis of covariance models using the contrast method. The estimates of the changes within groups were also calculated. Significance was set at $P < 0.05$ for all analyses. The same statistical analysis software (SAS System for Windows, version 9.3, SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses of biomarkers.

4.7.2 Experimental study (III)

A Friedman test was carried out to compare the scores of clinical outcomes over time for the four groups. A Dunn-Ferroni post-hoc test was performed when significant results were encountered. Change in clinical outcomes between CD and sham-operated joints were compared by independent-samples Mann-Whitney U-test. SPSS software was used for data analysis (SPSS Software, IBM, New York, NY, USA).

IL-1ra, PDGF-BB, TGF- β_1 , and TNF- α concentrations, WBC count, and TP concentrations were examined with analysis of covariance models. The study design had 3 time points and 2 different interventions (operation, treatment). The sample size was small and the two effects were analysed separately. The change in biomarker concentrations or WBC count from pre-operation to pre-treatment was examined in one analysis and the change from pre-treatment to end of follow-up in another analysis. In both analyses, the change of concentration was used as the response, the operation type or treatment as the fixed effect, and the corresponding baseline measurement as a covariate. For IL-1ra, PDGF-BB, TGF- β_1 , and TNF- α , a logarithmic transformation was applied to normalize the distributions.

Changes in biomarker and TP concentrations and WBC count were analysed also using Wilcoxon signed-rank test. The Wilcoxon test was conducted for both operations separately (for CD and for SO) and also for all 16 limbs (effect of operation regardless of type). Significance was set at $P < 0.05$ for all tests.

5 Results

5.2 Hyaluronan injection for treatment of pain originating from the metacarpophalangeal joint (I)

In 16 of the 30 horses, lameness had previously been localized in the same joint that was found to be lame at baseline. Altogether 15 of the 30 horses had received HA and/or corticosteroid treatment more than 3 months before entering the study in the same joint that was found lame at baseline. Nine horses had not received any medications previously for their musculoskeletal problems (Table 1).

5.2.1 Demographic variables and outcome measures at the baseline clinical examination

No significant differences emerged in signalment, use of the horse, outcome measures, or number of horses with findings in radiographs between the NASHA and placebo treatment groups at the clinical baseline examination. The baseline lameness score ranged from 1 to 3 (out of 0-5) and the most frequent lameness score was 2 ($n = 13$, 48%). In 7 horses (26%), the lameness score was 1 and in 7 horses (26%) 3 (Table 5).

5.2.2 Outcome measures after NASHA and placebo injection

When the change in the lameness score as the primary outcome measure was compared between the NASHA and placebo groups, no significant difference emerged ($P = 0.94$, Table 5). When the changes in the secondary outcome measures were compared, a significantly greater decrease of the response to the flexion test was recorded in the NASHA group ($P = 0.01$, Table 5). In contrast, the change in effusion and pain in flexion were not significantly different between the placebo and NASHA groups ($P = 0.94$ and $P = 0.27$, respectively, Table 5). All results were similar when also the three horses with metatarsophalangeal joint problems were included in the analyses.

Adverse effects in horses after NASHA and placebo injection

No adverse effects of either IA NASHA or saline injection could be seen clinically in any of the horses.

5.2.3 Follow-up

Based on the follow-up telephone interview at 2.5–3 months, 14 of the 21 horses (67%) had returned to their previous level of use, but two of these horses later developed other musculoskeletal problems: suspensory ligament injury (n=1) and OA of another joint (n=1). Six horse owners could not be reached for the follow-up interview. Seven of the 21 horses (33%) were no longer used in the same discipline or at the same performance level.

Table 5. Medians or means (for concentrations) with ranges in parentheses of outcome measures in the placebo and NASHA groups, with *P*-values for the within-groups comparison of outcome measures between the baseline and the clinical (end) examination, and *P*-values for the comparison between groups for the change in outcome measures after the clinical examination.

Outcome measure	Placebo baseline	Placebo end	<i>P</i> within placebo	NASHA baseline	NASHA end	<i>P</i> within NASHA	<i>P</i> between groups
Lameness ^a	2 (1-3)	2 (0-3)	0.005	2 (1-3)	0 (0-3)	0.016	0.943
Effusion ^b	1 (0-3)	1 (0-3)	0.480	1 (0-3)	1 (0-3)	0.564	0.943
Flexion test ^c	3 (2-4)	3 (1-4)	0.068	3 (2-4)	1 (0-3)	0.002	0.014
Pain ^d	2 (1-3)	1 (0-3)	0.038	2 (0-3)	0 (0-2)	0.010	0.270
WBC count x 10 ⁹ /l	0.29 (0.06-0.51)	0.35 (0.04-0.8)	0.608	0.21 (0.05-1.05)	0.59 (0.13-1.45)	0.021	0.045
TP g/l	8 (6-18)	8 (4-12)	0.002	10 (8-17)	10 (7-17)	0.271	0.136
PGE ₂ pg/ml	70 (44-3459)	31 (65-1883)	0.027	106 (43-2394)	60 (0-777)	0.010	0.768
Substance P pg/ml	15.8 (10.6-38.3)	13.7 (10.5-37.3)	0.328	13.1 (11.1-33.3)	13.6 (10.6-21.5)	0.826	0.662
CS846 pg/ml	2162 (1193-3303)	2061 (1204-3231)	0.385	2816 (1114-4117)	1924 (1117-3763)	0.010	0.207
CPII pg/ml	2273 (365-9376)	1033 (323-11194)	0.009	946 (2124-6061)	984 (400-4667)	<0.001	0.155

^a American Association of Equine Practitioners' (AAEP) scale 0–5

^b 0 = no effusion, 1 = mild, 2 = moderate, 3 = severe effusion, 4 = severe swelling of the metacarpal joint region

^c 0 = no increase, 1 = slight increase, 2 = moderate increase, 3 = considerable increase in baseline lameness, 4 = non-weight-bearing lameness

^d 0 = no pain at flexion, 1 = mild pain, 2 = moderate pain, 3 = severe pain

WBC, white blood cell; TP, total protein; PGE₂, prostaglandin E₂; CS846, aggrecan chondroitin sulphate 846 epitope; CPII, carboxypeptide of type II collagen

5.3 Experimental study: Clinical outcomes of experimental horses (III)

Five days after the arthroscopy (induction of CD or SO), all eight included horses showed lameness of the limb with the created CD in the intercarpal joint, increased score in the flexion test, and effusion in the joint of the affected limb. The mean lameness score in the CD group was 2.5, the mean flexion test score was 2.9, and the mean effusion score was 2.1. Two horses showed bilateral lameness, i.e. were mildly lame also in the SO limb. In the SO group, five days after the arthroscopy, mean scores of the flexion test and effusion were 1 and 2, respectively. No pain was elicited while keeping the carpus in maximal flexion in any of the horses.

In the lameness score of the CD+NASHA+ group, a significant difference was found between day 0 and day 5 ($P = 0.024$). A significant difference between day 0 and day 5 was found also in the effusion score of the CD+NASHA+ group ($P = 0.040$) and the SO+NASHA- group ($P = 0.040$) (Figure 2). When the effect of CD was examined alone, the lameness score and the flexion test score increased significantly ($P = 0.001$ and $P = 0.026$, respectively) only following the CD (i.e. between day 0 and day 5). The effusion score increased significantly between day 0 and day 5 following both the CD ($P = 0.005$) and sham operation ($P = 0.002$). Following the CD, on day 14, the flexion test and the effusion scores were still significantly higher ($P = 0.026$ and $P = 0.012$, respectively) than the score at baseline (day 0). When these groups were compared, at day 5 the lameness score and the flexion test scores were higher in the CD joints ($P < 0.001$) than in the sham-operated joints ($P = 0.038$).

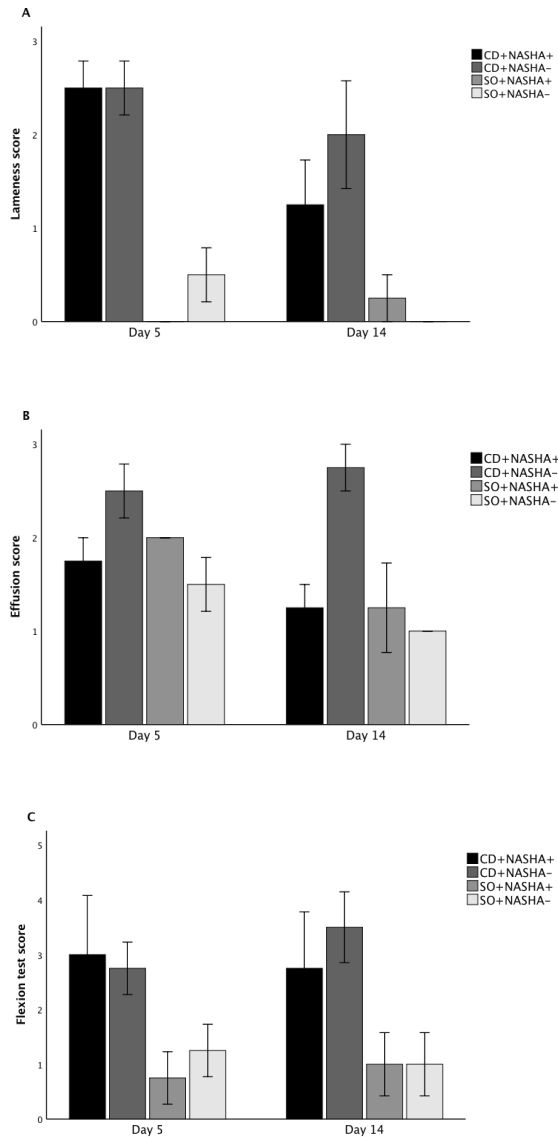


Figure 2. Mean (\pm SEM) clinical outcome measures of treatment groups post-operation (day 5) and post-treatment (day 14). CD+NASHA+: cartilage defect joints with NASHA injection; CD+NASHA-: cartilage defect joints without NASHA injection; SO+NASHA+: sham-operated joints with NASHA injection; SO+NASHA-: sham-operated joints without NASHA injection.

5.4 Synovial fluid biomarkers in arthritis and following NASHA injection (II,III)

Background variables (age, gender, breed, use of the horse, lameness score, duration of clinical signs, previous incidences of the affected joint, radiographic findings) did not have any effect on the concentration changes of the different biomarkers (II).

Synovial fluid white blood cell count and total protein concentration (II,III)

In the clinical study, NASHA induced an increase in the SF WBC count relative to baseline ($P=0.021$), and the same was seen in the placebo group ($P=0.045$) (Table 5) (II). In the experimental study, both the creation of the CD and the SO resulted in a significant increase in the WBC count ($P=0.011$ for CD and $P=0.002$ for SO) and TP concentration ($P=0.0002$ for CD and $P=0.002$ for SO) (III).

Prostaglandin E_2 (II)

When the change in SF PGE_2 concentration was compared between the NASHA and placebo groups, no significant difference was detected. When examining SF biomarkers within groups, the PGE_2 concentration decreased in both groups ($P=0.010$ for NASHA; $P=0.027$ for placebo) (Table 5).

Substance P (II)

When the change in SF substance P concentration was compared between and within the NASHA and placebo groups, no significant difference was detected (Table 5).

Markers of cartilage synthesis (II)

When the change in the SF CS846 concentration and the CPII concentration were compared between the NASHA and placebo groups, no significant difference emerged. When examining SF biomarkers within the groups, a significant decrease in the CS846 concentration from baseline was documented in the NASHA group ($P=0.010$), but not in the placebo group. The CPII concentration decreased in both groups ($P<0.001$ for NASHA; $P=0.009$ for placebo) (Table 5).

Interleukin 1 receptor antagonist (III)

No statistically significant effects of the operation or treatment on changes in IL-1ra concentration were detected, but significant differences between the time points emerged within the groups; the change from pre-operation to pre-treatment values was significant for IL-1ra ($P=0.034$ for the CD group and $P=0.010$ for the SO group) (Figure 3).

Growth factors (III)

No statistically significant effect of operation type or treatment on changes in PDGF-BB or TGF- β_1 concentration was detected (Figures 4 and 5).

Tumour necrosis factor alpha (III)

No statistically significant effect of operation type or treatment on change in this biomarker was detected (Figure 6). However, arthroscopy caused a significant increase in TNF- α concentration ($P=0.039$) in all joints when groups were combined.

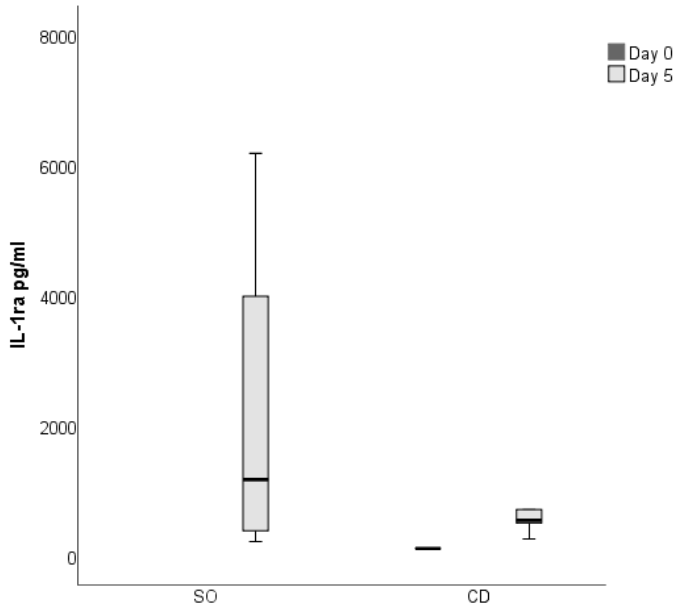


Figure 3. SF IL-1ra concentrations of SO and CD joints pre-operatively (day 0) and post-operatively (day 5). All concentrations of the SO group on day 0 were under the detection limit. The central horizontal line represents the median values and boxes indicate interquartile range (IGR). The top and bottom whiskers indicate the highest and lowest case within 1.5 times of the IGR, respectively. One outlier of more than 8000 pg/ml has been removed to clarify the figure). SO, sham operation; CD, cartilage defect.

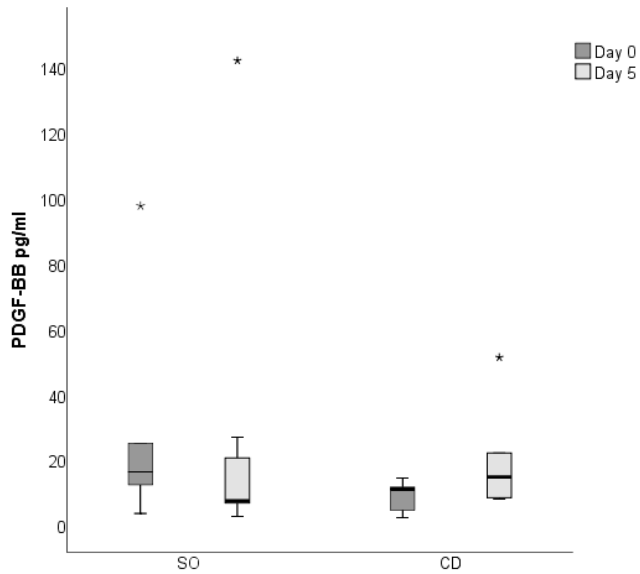


Figure 4. SF PDGF-BB concentrations of SO and CD joints pre-operatively (day 0) and post-operatively (day 5). The central horizontal line represents the median values and boxes indicate interquartile range (IQR). The top and bottom whiskers indicate the highest and lowest case within 1.5 times of the IQR, respectively. * = Values more than 1.5 times the IQR. SO, sham operation; CD, cartilage defect.

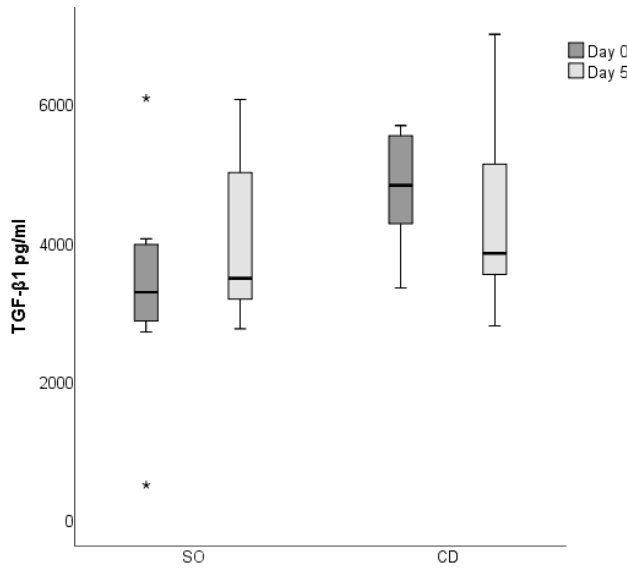


Figure 5. SF TGF- β_1 concentrations of SO and CD joints pre-operatively (day 0) and post-operatively (day 5). The central horizontal line represents the median values and boxes indicate interquartile range (IQR). The top and bottom whiskers indicate the highest and lowest case within 1.5 times of the IQR, respectively. * = Values more than 1.5 times the IQR. SO, sham operation; CD, cartilage defect.

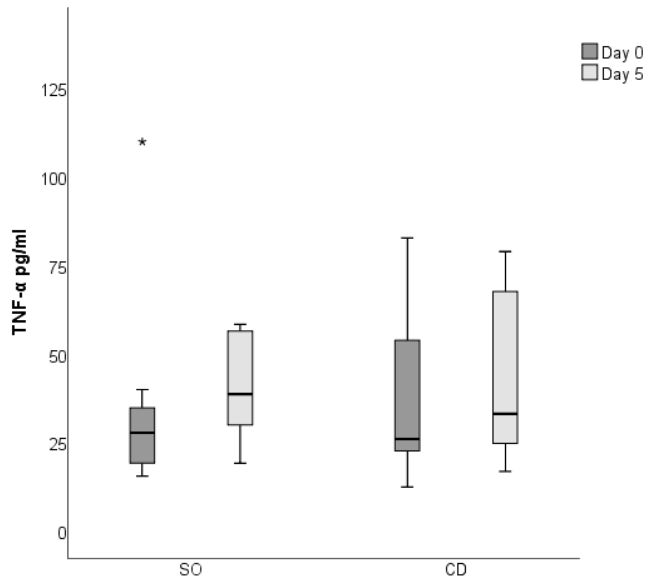


Figure 6. SF TNF- α concentrations of SO and CD joints pre-operatively (day 0) and post-operatively (day 5). The central horizontal line represents the median values and boxes indicate interquartile range (IGR). The top and bottom whiskers indicate the highest and lowest case within 1.5 times of the IGR, respectively. * = Values more than 1.5 times the IQR. SO, sham operation; CD, cartilage defect.

6 Discussion

6.1 NASHA injection for treatment of intra-articular pain

The hypothesis was that IA NASHA would relieve the clinical signs of joint inflammation compared with the placebo (I) or no treatment (III) in horses with synovitis or mild OA of the metacarpophalangeal joint (I) and in horses with an acute induced synovitis and CD of the intercarpal joint (III). Our results do not fully support this hypothesis; however, some symptom-modifying effects were documented.

The distal limb flexion test has been shown to be very specific for the disease and pain arising from the metacarpophalangeal joint (Kearney et al., 2010). In our clinical study on horses with lameness localized in the metacarpophalangeal joint, the flexion test score decreased significantly in the NASHA group compared with the placebo group, where the flexion test reaction persisted (I). As lameness score decreased along with flexion test score, horses could have been considered free of IA inflammation and pain after NASHA injection, whereas placebo-treated horses remained symptomatic. However, a significantly decreased lameness score was documented also in the placebo group, and the improvement in the lameness score of horses injected with IA NASHA was not bigger than in the placebo group. This is in contrast to the controlled clinical study on IA HA in horses (Gaustad and Larsen, 1995), where significantly improved lameness score in HA- and PSGAG-treated horses, compared with the placebo-treated group, was detected three weeks after the treatment. The larger sample size, the multiple joints involved, and the presence of different joint diseases in their study may explain the different results from our study. Furthermore, HA was administered there twice intra-articularly (instead of a single injection as in our study), the number of HA-treated joints was not reported, and both the HA- and PSGAG-treated horses were included in the same group.

Our findings are in accordance with those of Frisbie et al. (2009a), who reported a lack of improvement in the lameness score compared with control horses when HA was administered to horses with experimentally induced OA. However, our results considering flexion tests suggest that NASHA has pain-relieving properties. Furthermore, it is possible that improvement of lameness in the NASHA and placebo groups was evident because both NASHA and NaCl improved lameness, as NaCl has been shown to relieve lameness more than rest alone (Gaustad et al., 1999). In the experimental horses (III), our modified model of CD induced significant lameness, reaction in the flexion test, and effusion of the operated joint, the latter two still being increased on day 14, although clinical signs had improved in CD joints with NASHA (Figure 2). However, that improvement in the CD+NASHA+ group was not significant over time (between days 5 and 14) or relative to the CD+NASHA- group. In the experimental study, NaCl as a placebo treatment was not administered. Deterioration, albeit not statistically significant, of effusion and flexion test scores in the CD+NASHA- group was seen (Figure 2). This suggests that the

improvement in clinical signs detected in our studies resulted from symptom-modifying effects of NASHA (and to some extent from the effect of NaCl in the clinical study).

In a recent review, NASHA was reported to be superior or at least equivalent to the other IA HA treatments for knee OA in human patients in seven studies fulfilling the criteria for level I evidence, i.e. all were high-quality randomized controlled trials (Leighton et al., 2018). However, in another meta-analysis Johansen et al. (2016) reported that the benefit of IA HA in relieving pain in human OA patients was minimal, but potentially relevant when the HA group was compared with the saline group or the non-intervention control group. This study included also a few studies on NASHA. Based on our results, NASHA seems to alleviate pain to some degree also in lame horses, but it is likely that NASHA alone will not have a marked effect on clinical lameness two weeks after injection.

In horses, NASHA could be beneficial over the other IA HA products due to its long half-life and long residence time in the joint (Lindqvist et al., 2002; Åkermark et al., 2002; Altman et al., 2004) as well as its long-acting pain-relieving effects after a single injection (Åkermark et al., 2002; Altman et al., 2004; Goldberg and Buckwalter, 2005; Raman et al., 2008; Abate et al., 2010; Chevalier et al., 2010; Arden et al., 2014; Leighton et al., 2014). This would mean fewer treatments and decreased costs, along with less risks associated with repeated injections and less sport carrier disruptions due to doping withdrawal periods. In our study, 67% of horses were able to perform at their previous level three months after a single NASHA injection, but the long-term efficacy of NASHA in horses warrants further examinations.

6.2 Tolerability of NASHA

Any IA injection may cause adverse joint reaction with lameness, increased SF volume, and elevated WBC count and TP concentration (Goodrich, 2011). Clinically, no adverse effects of the IA NASHA injection were seen in any of the clinical patients (I,II) or experimental horses (III). However, the SF WBC count of the clinical patients increased significantly after IA NASHA compared with placebo. An IA injection of NASHA may induce a mild inflammatory response and signs of IA pain (Leighton et al., 2014).

The arthrocentesis was performed through the lateral sesamoidean ligament, which has been shown to induce less synovial haemorrhage than the procedure through the proximal palmar pouch (Misheff and Stover, 1991). Hence, blood contamination, although seen in some samples, is an unlikely explanation for the markedly increased WBC count. It is therefore likely that the inflammatory reaction in the joints was caused by the NASHA itself. A similar inflammatory response in horses has been reported after an IA injection of a combination of pentosane polysulphate and glucosamine injection in horses (Kwan et al., 2012). Also in humans, a transient IA inflammation has been noted, especially after consecutive HA injections (Leopold et al., 2002; Pullman-Moore et al., 2002; Marino et

al., 2006). Inflammation has been thought to result from a cell-mediated hypersensitivity reaction, induced by low MW breakdown products of HA (Pullman-Mooar et al., 2002; Marino et al., 2006). The incidence of local adverse events after IA NASHA in humans has been reported to be 5% (Åkermark et al., 2002) and 12.5% (Altman et al., 2004). These events involved pain, swelling, tenderness, or stiffness of the knee. No serious adverse reactions, such as acute severe IA inflammation or pain resembling IA sepsis, were encountered. It was speculated that the relatively high single dose (60 mg) and volume (3 ml) used may have caused a feeling of swelling and tension in the knee (Åkermark et al., 2002). Moreover, since all of the adverse events reported by patients involved signs experienced during the natural course of OA, such as localized pain, swelling, or stiffness of the knee, the relationship between the NASHA injection and these events was difficult to ascertain. In any case, NASHA seems to be relatively well tolerated by both human and, based on our study, equine patients.

6.3 Biomarker analyses

6.3.1 Effect of NASHA on synovial fluid biomarkers in lameness originating from the metacarpophalangeal joint (II)

Measured baseline concentrations of PGE₂ and substance P were slightly lower than in a previous study on equine SF (Kirker-Head et al., 2000). However, considering the diagnosed OA in the earlier study and the milder joint disease documented in the present study, the results are comparable. In contrast, baseline SF concentration of CPII was much higher here than in an earlier study on IA pain of metacarpophalangeal joints (de Grauw et al., 2006a). The reason could be the acute inflammatory process with increased cartilage matrix metabolism in our study population.

Contrary to our hypothesis, the change in biomarker concentrations was not greater in the NASHA group than in the placebo group. The PGE₂ concentration decreased significantly in both groups. The variability in PGE₂ concentration was high in the present study. This may result from high variations in responses to treatment; in the case of synovitis, SF PGE₂ concentration may have decreased more rapidly than in mild OA. A long-standing increase in SF PGE₂ concentration after arthroscopically induced OA has been reported (Frisbie et al., 2008), while the increase was shorter in other studies using the LPS inflammation induction model (Owens et al., 1996; de Grauw et al., 2009a). Based on these studies, variation in PGE₂ concentrations in our study is suggestive of variation in disease stages in the clinical study population. Bertone et al. (2001) stated that variability of PGE₂ concentration hindered discrimination among the types of equine joint diseases, although SF PGE₂ concentration was considered to be a good or excellent predictor of joint disease and a functional screening test tool in horses. However, a lack of treatment effect by HA on SF PGE₂ concentration was also reported in experimental

studies with more uniform osteoarthritic joints (Frisbie et al., 2009a; Frisbie et al., 2013). IA NaCl can alleviate lameness related to IA inflammation (Gaustad et al., 1999). In the present study, both NASHA and NaCl may have relieved IA inflammation and decreased SF PGE₂ concentration, resulting in the lack of treatment effect of NASHA.

In the present study, no differences in SF substance P concentrations were observed within or between treatment groups. This is in contrast to the reported elevated SF concentrations in osteoarthritic joints compared with normal equine joints (Kirker-Head et al., 2000). Substance P concentration has been shown to be associated with joint pain (de Grauw et al., 2006a). In the equine LPS-induced synovitis model, SF substance P concentration decreased following peroral meloxicam treatment (de Grauw et al., 2009b), but not after IA opioid analgesia (van Loon et al., 2010). Both medications resulted in decreased lameness. The differences in responses after these medications can be explained by different mechanisms of action, i.e. opioid analgesia has presynaptic control, while NSAIDs influence the release of substance P (de Grauw et al., 2006a; de Grauw et al., 2009b). The complete mechanism of action of HA in the joint remains unknown. The clinical section (I) of this NASHA study revealed a reduction in pain sensation. Besides protecting from mechanical forces (Gomis et al., 2004) and modulating pain receptor channel opening (Caires et al., 2015), the binding of neuropeptides could be one pain-reducing mechanism of HA. The results of the present study suggest, however, that intrasynovial NASHA injections do not affect substance P concentration in SF.

CS846 can be considered a promising indicator of inflammation-induced cartilage turnover. An increase of SF CS846 has been described after surgically induced OA (Koenig et al., 2014) and LPS-induced synovitis (de Grauw et al., 2014) in horses and also following naturally occurring injury and OA in humans (Lohmander et al., 1999). The increase of SF CPII and CS846 concentrations after IA lidocaine and bupivacaine in healthy equine joints has been suggested to indicate the reparative response to the short chondrotoxic effect of these compounds (Piat et al., 2012). Not only harmful compounds or surgery but also exercise induces an elevation of SF CS846 concentration in horses (Frisbie et al., 2008). In the present study, SF CS846 concentration decreased following IA NASHA injection. This, as well as a decrease in SF CPII concentration, can be interpreted as decreased inflammation and turnover of articular cartilage matrix, meaning that less damage, and hence, less cartilage repair has occurred post-injection. However, a similar effect was not reported in the experimental study, where a combination of sodium pentosan polysulphate and N-acetyl glycosamine was administered intra-articularly (Kwan et al., 2012).

However, SF CPII concentration decreased significantly in both the NASHA and placebo groups. The decreasing concentrations of SF CPII after IA injections in our study could also indicate compromised synthesis of type II collagen. On the same day, before IA NASHA or saline (placebo) injections, IA mepivacaine was injected in all joints due to diagnostic analgesia. Although a prolonged decrease of SF CPII has been reported after IA methylprednisolone in horses and suggested to result from the inhibition of collagen synthesis (Robion et al., 2001), it is unlikely that mepivacaine had such a long-standing

effect. Furthermore, it seems unlikely that mepivacaine disturbs the type II collagen synthesis since related compounds lidocaine and bupivacaine have been shown to have the opposite effect (Piat et al., 2012), as discussed above. More plausible is that the decrease in SF CPII concentration indicated less degradation, and hence, also less repair of cartilage in compromised joints after the horses had discontinued training and rested while participating in this study.

As described above, all immunoassays used in the study have been used for analysis of equine SF. The structure of arachidonic acid derivatives is similar among species, so a PGE₂ assay can also be applied to samples obtained from horses (Bertone et al., 2001). Because equine substance P is identical to human substance P (Studer et al., 1973), an assay originally intended for use in human biological fluids was employed in equine samples. Cross-reactivity can constitute an analytical problem in immunoassays. The manufacturer of the commercial substance P immunoassay used in the study reported 100% cross-reactivity between substance P and a novel neuropeptide hemokinin-1 in human samples in their assay. However, in contrast to substance P, hemokinin-1 is mainly expressed in non-neuronal tissues (Bellucci et al., 2002). To the best of our knowledge, hemokinin-1 has not been described in horses, and thus, it is not currently known whether the cross-reactivity applies to horses.

6.3.2 Effect of induced cartilage defect and NASHA on synovial fluid biomarkers (III)

Concentrations of the measured biomarkers in intact joints were fairly similar to those in a previous report (Ríos et al., 2015a). IL-1ra, PDGF-BB and TGF-β₁ have mostly been documented in equine articular tissues *in vitro* (Iqbal et al., 2000; Ríos et al., 2015a; Ríos et al., 2015b; Ríos et al., 2015c).

IL-1ra concentration in the SF has been shown to increase after an acute IA fracture in humans. However, no difference in SF inflammatory cytokine concentration between high- and low-energy injuries was detected (Haller et al., 2015). Although not directly comparable with IA fractures, in the present study no difference was detected between CD joints and SO joints where the arthroscopy itself caused trauma to the synovial soft tissues and may have caused the increase of IL-1ra concentration. Blocking the IL-1β receptor by IL-1ra has potentially a wide positive effect on inhibiting deleterious events in OA (Roman-Blas and Jimenez, 2006).

After sampling at baseline on day 0, the next sampling was performed on day 5, at a time point where PDGF-BB is supposed to play a major role in vascular formation and proliferation of fibroblasts in ongoing repair (Anitua et al., 2009). Five days after CD, SF PDGF-BB concentration was found to be increased in CD joints, albeit not significantly, suggesting that the increase of PDGF-BB in SF has occurred earlier in the course of injury and inflammation. In contrast to acute inflammation, PDGF-BB concentration of human OA joints has been reported to be under the detection limit or to equal the concentration of

healthy joints (Beekhuizen et al., 2013; Mabey et al., 2014), which is also suggestive of an early increase in SF PDGF-BB concentration and its role in the initial phase of the pathogenesis of OA.

In this study, neither the creation of a CD nor the IA NASHA induced an increase in SF TGF- β_1 concentration. In contrast, TGF- β_1 concentration decreased following CD, although no significant difference between CD and SO joints was found. This contradicts a previous study on equine joints, which reported an increase of SF TGF- β_1 concentration challenged with LPS (Carmalt et al., 2011). One explanation can be the dilution effect of SF in CD joints due to induced inflammation and subsequent synovitis.

Ríos et al. (2015b) reported an increase in the TGF- β_1 concentration after a LPS challenge in an *in vitro* model of cartilage inflammation. The effect was suggested to result from a possible anti-inflammatory mechanism or by direct damage induced by LPS in the cartilage. A similar effect has been seen after LPS challenge of synovial membrane explants *in vitro* (Ríos et al., 2015a). The lack of increase in SF TGF- β_1 concentration in the present study is more consistent with the study of human OA, where SF TGF- β concentration was measurable in healthy subjects, while in patients with OA the concentration of SF TGF- β was low or even undetectable (Blaney et al., 2006). Cell signalling pathways may be intercepted by the inflammatory cytokines, diminishing the amount of TGF- β over the course of OA (Derynck and Zhang, 2003).

TGF- β_1 has an anabolic effect on cartilage; it is able to induce chondrogenic differentiation of mesenchymal stem cells, with rapid biosynthesis of glycosaminoglycan and deposition of extracellular matrix (Barry et al., 2001; Tuli et al., 2003). However, enhanced expression of TGF- β_1 has been associated with developing osteophytes (Scharstuhl et al., 2002) and hyperplasia of the synovium (Backer et al., 2001). It has been suggested that only a narrow concentration range of bioactive TGF- β is beneficial for cartilage health, and any concentration below or above this range may cause aberrant alterations in the TGF- β pathways, resulting in abnormal cartilage function (Finnson et al., 2012). In the present study, no changes in the SF TGF- β_1 concentrations were documented after induction of inflammation or after NASHA treatment, possibly implying that optimal SF TGF- β_1 concentrations were already present in the joints.

The results of studies on equine and human TNF- α in SF and in other IA tissue are somewhat contradictory. However, it seems to be a sensitive but not very specific marker of IA insults. The results of our experimental study are also consistent with this. The TNF- α concentration increased in the SF after induction of CD, although significant differences were not detected between the small population groups. An increased concentration of SF TNF- α has been documented in naturally occurring OA in equine carpi (Kamm et al., 2010) and in LPS-induced inflammation in equine joints (Hawkins et al., 1995; Carmalt et al., 2011). In contrast, LPS did not induce equine chondrocytes and synoviocytes to produce TNF- α *in vitro* (Armstrong and Lees, 2002). Furthermore, TNF- α was not shown to be a useful biomarker in different types of joint lesions in a clinical equine study (Ley et al., 2007). Similarly, in humans, increased levels were not associated with any particular

type of joint disease, although in OA patients detectable SF levels of TNF were associated with a long duration of joint disease (di Giovine et al., 1988). Furthermore, in horses, exercise alone leads to a significant increase in SF TNF- α concentration for a short period (Billinghurst et al., 1995; van den Boom et al., 2004). However, an increase in SF TNF- α concentration as a result of serial arthrocenteses has not been demonstrated (Billinghurst et al., 1995). Contrary to this, gas and liquid capsular distension of arthroscopied joints provoked an inflammatory response with an increased concentration of SF TNF- α (Rossetti et al., 2012). Platelet-rich gel has been shown to induce TNF- α release in LPS-challenged equine synovial membrane and cartilage explants *in vitro* (Ríos et al., 2015a; Ríos et al., 2015b), but a similar increase in TNF- α concentration has not been reported *in vivo* after PRP injection in healthy equine joints (Moraes et al., 2015). Induction of CD in the present study was likely adequate to cause marked IA inflammation, and thus, probably with a larger sample size and/or more frequent sampling a significant increase due to the operation would have been detected.

After a mild increase in biomarker concentrations resulting from arthroscopy and induction of synovitis, NASHA failed to produce any further effect. The CD model of the present study may have been insufficient in induction of powerful IA inflammation, as lameness score and presumably also increase in SF biomarker concentrations were relatively low compared with those of LPS induction models (de Grauw et al., 2009a; de Grauw et al., 2009b; de Grauw et al., 2014). This may have resulted in a lack of significant treatment effects of IA NASHA. However, it is also possible that NASHA does not have effect on the SF biomarkers measured, although HA has been shown to have an anti-inflammatory effect (Gomis et al., 2004; Frisbie et al., 2009a). However, IA HA injections have also been reported to induce a transitional IA inflammatory reaction with pronounced clinical signs of inflammation and pain (Marino et al., 2006). To our knowledge, only one study has explored biomarkers in equine SF after IA HA (Frisbie et al., 2009a). As HA is frequently used in IA treatment of horses, its mechanism of action warrants further research.

6.4 Study limitations

A considerable limitation in the clinical (I, II) and experimental studies (III) was the small sample size. The lameness score as an outcome parameter directed the calculation of sample size in the clinical study, and the expectations on proportions of improved (measured with the AAEP lameness scale) horses in the placebo and NASHA groups had to be based on human studies and on the few existing clinical equine studies on IA HA. As described, the proportion of improved horses in the placebo group was expected to be 20-30% based on proportions reported in human studies (Richette et al., 2009; Pham et al., 2004) and in the NASHA group 87% based on proportions in an equine study on HA for the treatment of naturally occurring arthritic conditions (Ruth and Swites, 1985). It would

have been better to base also the proportion of improved placebo-treated horses on equine studies, however, to the best of our knowledge, only one clinical study with IA saline as a placebo exists (Gaustad and Larsen, 1995), and that study has the same portion (30%) of improved horses (at two weeks) as used in our calculations. Furthermore, also the proportion of improved horses, based on that reported in the early clinical study on IA HA (Ruth and Swites, 1985), was probably estimated to be too large, and we had smaller differences between improvement rate of groups and lower statistical power than presumed. In addition, although the AAEP lameness scale has distinct criteria and differences between grades and has been used as a measure of improvement in equine studies (de Grauw et al. 2009a; de Grauw et al., 2009b); Frisbie et al., 2009a; Frisbie et al., 2013), improvement of one grade may have been insufficient to detect differences between the groups in the present study.

Although the number of horses in the experimental studies on equine joints generally ranges from 6 to 12 (Hawkins et al., 1995; Frisbie et al., 2002; de Grauw et al., 2009a; Carmalt et al., 2011; Rossetti et al., 2012), a sample size of 8 horses (16 joints) in the experimental study (III) was likely too low to detect differences in the measured SF biomarker concentrations. For the experimental study, the number of horses financially possible to include were included. However, when calculating the sample size with the formula $n = ((z \cdot \sigma) / \text{MOE})^2$, where z is value from the standard z -distribution, σ is standard deviation, and MOE is the margin of error, with values reported for equine SF IL-1ra in another study (Ríos et al., 2015a), a sample size of ~6 is achieved. However, the deviation in SF IL-1ra concentrations in inflamed joints of the present study was higher and the bigger sample size would have been needed.

The severity of the joint disease in the clinical study population was variable, despite the uniform baseline lameness and other clinical scores between the groups, resulting in varying response of clinical signs to IA NASHA treatment. Furthermore, as the disease stage and the intensity of inflammation affect concentrations of cartilage-derived markers (Johnson et al., 2002), SF biomarker concentrations were highly variable within the groups along with suspected more acute inflammation and/or advanced cartilage matrix degradation in some of the joints. High variation in biomarker concentrations as well as high intra-assay CV% in some of the markers may also have resulted in a lack of power for detecting a significant difference between the groups. As viscous SF is difficult to handle with the pipet, the deviation of results may potentially be high, especially in the case of low CS846 concentrations. To overcome this, samples were diluted and analysed in triplicate. If the CV% was over 20, samples were analysed again with three parallel samples whenever possible, which all yielded CV% < 20. However, in some samples this was not possible (not enough SF left), and the deviant result was rejected and results were based only on two parallel analyses with CV% < 20. In serological assays, a twofold difference in measurements of the same sample has been widely regarded as the upper limit of acceptable variability. Having CV% < 20 as a limit for rejection, already the one twofold difference suggests that interference is evident in the assay, i.e. values represent true errors, not random variation that is always present in sample replicate ELISAs (and

that cannot be removed as an outlier) (Reed et al., 2002). While horses with uniform breed and sport discipline were selected for the experimental study and all horses were free of lameness and intercarpal joint disease, differences in exercise or training status and age may have caused variation in baseline SF biomarker concentrations.

The model in the experimental study has not previously been used in this modified version, but it was planned to mimic clinical disease and osteochondral changes encountered in Standardbred horses (Steel et al. 2006; Bertuglia et al., 2016). We did not want to use the established chip model with longitudinal training since the long-term maintenance of horses was not possible in this study. Instead, we wanted to establish a more clinically relevant, defined cartilage injury on the third carpal bone, often seen in clinical cases of Standardbred trotters, to examine the short-term effects of NASHA on the clinical signs and synovial fluid parameters of the arthritic joints. Also, disease in the middle carpal joint without a chip is a common clinical disease (Steel et al., 2006). The carpal lameness related to the middle carpal joint has been reported to comprise almost a third of the young Standardbred racehorses, and to be present in 56% of those horses with forelimb lameness (Steel et al.; 2006). Chemically induced IA inflammation has regularly been used in equine models (Owens et al., 1996; de Grauw et al.; de Grauw et al. 2009a; de Grauw et al., 2009b; Carmalt et al, 2011; de Grauw et al., 2014). However, although they may be used in animal models for studying OA pain-related behaviours in general (Gregory et al., 2012; Malfait et al., 2013), their validity as clinical models for OA has been questioned, mainly because of the widespread cell death and rapid joint destruction, which do not resemble either spontaneous or post-traumatic OA (Poole et al., 2010; Teeple et al., 2013). As the surgery used in the present study induced clinical signs and increase in SF IL-1ra concentration that the sham operation did not, the modified model of carpal OA could be considered successful and adequate for studying post-traumatic OA.

Subjective scoring of lameness with the AAEP scale or another subjective scale has traditionally (Frisbie et al., 2002; Frisbie et al., 2007; Frisbie et al., 2008; Frisbie et al., 2009a; Frisbie et al., 2009b; Frisbie et al., 2013; de Grauw et al., 2007; de Grauw et al., 2009a; de Grauw et al., 2009b; de Grauw et al. 2014) and is still being used in equine studies (Giunta et al., 2019; Smit et al., 2019). However, intra- and inter-rater reproducibility (although usually not reported in equine studies) is not very high (Fuller et al., 2006); Hammarberg et al., 2016) when repeated scorings are used, increasing uncertainty in the results. Lameness evaluation could be improved by using more objective measurements (Rhodin et al., 2013; Greve et al., 2017), but they were not available to us at the time of this study. However, a recent study on an inertial sensor system during clinical examination of hindlimb lameness in horses reported only fair agreement between a highly experienced group of veterinarians and the inertial sensor system (Leelamankong et al., 2019). In addition to the objective lameness scoring, goniometry for measuring the angle of joint in maximal flexion (Adair et al., 2016) and measurement of joint circumference could add accuracy to clinical scorings. Despite limitations due to the lack of objective measurements in the clinical examinations here, a

strength in the clinical scoring is that all of the scoring was done by the same experienced veterinarian who was blinded to the treatments in the clinical study.

The SF sampling time points may not have been ideal for all of the measured biomarkers (II,III). SF is also a constantly changing media; synovitis influences the volume of SF, and thereby, also the biomarker concentrations may change rapidly. Finding an optimal time point for SF aspiration is challenging, especially when several biomarkers are studied. In some of the clinical patients (II), SF biomarker concentrations may have returned close to baseline, while others may have had increased SF biomarker concentrations, as discussed earlier, due a more acute IA disease. Therefore, changes in SF marker concentrations are challenging to test and the results are often contradictory, as documented in many of the previous studies cited. Furthermore, an optimal time point for SF sampling after IA NASHA may have been earlier than two weeks. Since a maximum effect for NASHA treatment has been reported to be six weeks post-treatment in human knee OA patients (Altman et al., 2004) and for HA three weeks post-treatment in equine synovitis patients (Gaustad and Larsen, 1995), the post-treatment sampling and evaluation time point of two weeks in the clinical study (I,II) was selected. As the study population consisted of client-owned horse patients and many had to travel a long distance to the hospital, it was logistically difficult to organize additional SF sampling visits before or after two weeks. On the other hand, IA injection itself, especially when conducted repeatedly, can result in increased SF biomarker concentrations (Brama et al., 2004). Before subsequent SF collection, a 14-day rest period after the previous arthrocentesis has been recommended to minimize this effect (van den Boom et al., 2004).

To summarize, a time period of two weeks between the first and second clinical examination and SF sampling was chosen to optimize both the timing of sampling and the clinical effect of NASHA (not too early to avoid the potential effect of first arthrocentesis on biomarkers and to give time for NASHA to act). Rest for one to two weeks after IA HA injection is usually recommended, and we wanted to mimic and document effects of that in the study. Furthermore, we wanted to avoid withholding treatment of client-owned horses for an unnecessarily long period. However, had financial and time constraints (both the Department of the Small Animal and Equine Medicine and the horse owners) been overcome, additional SF samplings at weekly intervals after the IA NASHA and placebo would have been beneficial.

In the experimental study (III), the SO complicated the choice of the optimal sampling time point. The arthroscopy procedure as well as harvesting the synovial tissue samples cause mild trauma to the synovial soft tissues and also haemarthrosis. These may also have affected the concentrations of measured markers. However, the effect was thought to be transient, and to minimize the impact of arthroscopy and the sampling of synovial soft tissue, the sampling of SF and the IA HA injection were performed at five days as a balance between a too early and a too late intervention time point. Furthermore, no signs of blood in SF samples were seen. It would have been interesting to follow the concentrations of selected markers for a longer period. Due to financial constraints, however, this was beyond the scope of the study.

In the experimental study, the contralateral limb of the horse served as a control. It has been reported that concentrations of cartilage matrix products are also elevated in the contralateral knee in human patients with anterior cruciate ligament rupture, possibly as a consequence of altered loading (Dahlberg et al., 1994). Cytokines and degraded matrix products released from an operated joint may also be transported to the contralateral joint by the circulation and initiate an inflammatory process there as well. This was possible also in the present study. Measurement of serum concentrations of studied markers, as well as additional pro-inflammatory markers and markers of cartilage matrix metabolism, would have provided more information to test this hypothesis. It was, however, beyond the scope of this study. Using the sham-operated contralateral joint as a control may interfere with the interpretation of concentrations of SF biomarkers and clinical signs.

6.4 Future perspectives

The true value as well as the mechanism of action of IA NASHA in relieving inflammation and pain in arthritis and osteoarthritis remains to be fully established. The effect of IA NASHA on the selected SF pro-inflammatory, anti-inflammatory, and anabolic biomarkers in different types of equine arthritides warrants further research.

Most therapeutic agents used in OA have given rather inconsistent results with various biomarkers, most likely related to the absence of proven DMOADs (Lotz et al., 2013; Hosnijeh et al., 2019). Moreover, the selection of appropriate biomarkers to test treatment effects is crucial. While ELISA analyses did not reveal an effect of IA NASHA on most of the SF biomarkers examined here, more sophisticated techniques, such as SF proteomics, could yield biomarkers more suitable for investigating the effects of NASHA on inflammation, pain, and metabolism in a joint. Proteomics comprises the comprehensive profiling of protein contents. The proteomic profile of the SF is dependent upon the articular disease and its characterization has allowed the differentiation of OA from rheumatoid arthritis in humans (Mateos et al., 2012) and the differentiation of OA from septic arthritis (Anderson et al., 2019) and normal joints (Peffer et al., 2015) in horses.

In addition to detecting proteins associated with OA and discovering new biomarkers, proteomic analysis of SF, cartilage, and serum has been used recently for elucidation of the pathophysiology of OA and monitoring of orthopaedic diseases also in horses along with other animal models (Chiaradia et al., 2012; Garner et al., 2013; Peffer et al., 2015; Shadid et al., 2018; Skiöldebrand et al., 2017). In equine SF, protein variations related to inflammation, coagulation, oxidative stress, and matrix damage pathways have been identified (Chiaradia et al., 2012; Peffer et al., 2015), and they were suggestive of pathological alterations in articular homeostasis, plasma-SF exchange, joint nutritional status, and vessel permeability (Chiaradia et al., 2012). Moreover, especially for diagnostic and monitoring purposes, identification of specific SF components (such as COMP neopeptide) in OA is useful, as SF is closely associated with degenerating articular cartilage, the primary site for OA progression (Ritter et al., 2013; Peffer et al., 2015; Skiöldebrand et al., 2017). In addition, proteomic analysis of SF has been used to study

treatment effects of ACS in equine OA, and six deregulated proteins were suggestive of an improvement of joints (Chiaradia et al., 2012).

Moreover, reverse transcription polymerase chain reaction (RT-PCR) analysis of synovial tissues could be considered in investigating the biomarkers of the present studies as well as the treatment effect of IA NASHA. Increased expression of TNF- α , IL-1 β , aggrecanase 2, and matrix metalloproteinase 13 has been reported in the osteoarthritic equine joints (Kamm et al., 2010). Even peripheral WBCs have been suggested to have the potential for OA diagnostics in horses, as a correlation between the gene expression profile in WBCs, cartilage, and synovium and the cartilage turnover proteins in induced carpal OA was detected (Kamm et al., 2013).

A clinical trial after IA NASHA with a longer SF sampling period would be of interest. As in human studies, the maximum clinical effect has been reported to occur after a fairly long period, six weeks after the IA dosage of NASHA (Altman et al., 2004), it would be worthwhile to follow the effect of NASHA on both clinical signs and SF biomarkers up to 6-12 weeks. Moreover, despite a reported delayed treatment effect on clinical signs, the immediate effect of NASHA on SF biomarkers after experimentally induced IA inflammation warrants further investigations.

To improve the evaluation of pain and lameness in equine OA research, the use of objective methods should be considered; measurement based on inertial sensors has proven to be a relatively reliable method (Greve et al., 2017).

7 Conclusions

Regarding our four objectives, the following conclusions can be drawn:

1. A single IA NASHA injection was not superior to an IA saline injection in reducing lameness in horses with synovitis or mild OA in the metacarpophalangeal joint. However, NASHA may have some beneficial effects in modifying clinical signs.
2. Although the cartilage-derived biomarker CS846 concentration in SF decreased only in the NASHA group, no significant change of any of the biomarkers was observed between the two treatment groups. A single IA injection of NASHA may be useful in the treatment of acute synovitis, but more research is needed on the effects of NASHA in other equine joint diseases as well as on its mechanism of action.
3. The experimental study demonstrated that arthroscopy and both the creation of CD and SO led to an increase in SF IL-1 α and TNF- α concentrations, while changes in concentrations of anabolic growth factors TGF- β_1 and PDGF-BB in SF were not seen five days after the arthroscopy. NASHA did not have an effect on the concentrations of the selected biomarkers.
4. In the clinical study, the IA NASHA injection induced a significant increase in SF WBC count; this may indicate an inflammatory response akin to that reported in human studies. However, IA NASHA injection was well tolerated, as clinically no adverse effects were observed either in the clinical study or in the experimental study.

As an overall conclusion, we can state that in a short-term follow-up IA NASHA does not relieve lameness, but seems to slightly improve clinical signs such as reactivity in the flexion test. Furthermore, NASHA over a short period does not significantly change the metabolic or inflammatory state of the joint.

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